European Society of Human Reproduction and Embryology

Course 4

Special Interest Groups "Embryology"

"In vitro culture conditions for human gametes and embryos: Present and future"

27 June 2004

Berlin - Germany

Evaluation Form

Course 4 - Embryology

27 June 2004 Berlin/Germany

DEMOGRAPHICS

Are you a physician? ___ Yes ___ No

Years since completion of training: ___ less than 5 ___ 5-9 ___ 10-19 ___ 20/over ___ currently in training

GENERAL INFORMATION	*Strongly* Agree	*Moderately* Agree	*Moderately* Disagree	*Strongly* Disagree
1. The course objectives were clearly stated.	_____	_____	_____	_____
2. The course objectives were clearly met.	_____	_____	_____	_____
3. Faculty/participant interaction was satisfactory.	_____	_____	_____	_____
4. The course was well organized.	_____	_____	_____	_____
5. Site accessibility was not a problem.	_____	_____	_____	_____
6. Meeting facilities were adequate.	_____	_____	_____	_____
7. Course director conducted program well.	_____	_____	_____	_____
8. Overall course grade (circle one)	A	B	C	D
9. Overall syllabus grade (circle one)	A	B	C	D

EDUCATIONAL VALUE

1. I learned something new that was important	_____	_____	_____	_____
2. I verified some important information	_____	_____	_____	_____
3. I plan to discuss some of this information with my colleagues.	_____	_____	_____	_____
4. I plan to seek more information on this topic	_____	_____	_____	_____
5. My attitude about this topic changed in some way	_____	_____	_____	_____
6. This course is likely to have an impact on my teaching	_____	_____	_____	_____

FACULTY EVALUATIONS

Strongly Agree = 4 Moderately Agree = 3
Moderately Disagree = 2 Strongly Disagree = 1

	MERMILLOD	MIKKELSEN	DURAND	TESARIK	DE JONGE	BARRATT	LEESE	MENEZO	KOLIBIANAKIS	GARDNER
1. Sufficient depth										
2. Concepts clearly explained										
3. Enhanced understanding of key matters										
4. Without commercial bias										
5. Slides readable										
6. Learning objectives clear										
7. Helpful for future reference										

COMMENTS

1. What change(s), if any, do you plan to make in your teaching as a result of this course?_____
   _____
2. Future suggested meeting topics and speakers? _____
   _____
3. Additional comments _____
   _____

OPTIONAL Would you be willing to be contacted in the future regarding how this course influenced your practice?

YES **NO**

Name _____ Phone _____

E-mail _____

Content

Program

Sunday, 27 June – Hall 8
Course 4: SIG Embryology Pre-congress Course on "In Vitro Culture Conditions for Human Gametes and Embryos: Present and Future"

Course co-ordinators: D. Royere (F), G. Hartshorne (UK), E. Van den Abbeel (B)

Topic 1: In vitro culture conditions for gametes

In vitro maturation of oocytes
09.00 - 09.30: Basic requirements - P. Mermillod (F)
09.30 - 10.00: Clinical experience - A.L. Mikkelsen (DK)

In vitro maturation of male germ cells
10.00 - 10.30: Which end-points are required? - P. Durand (F)
10.30 - 11.00: Clinical experience - J. Tesarik (E)
11.00 - 11.30: Coffee break

Topic 2:In vitro culture conditions for fertilization

Capacitating media
11.30 -12.00: Basic requirements - C. De Jonge (USA)
12.00 -12.30: Clinical aspects – C. Barratt (UK)

12.30 - 13.30: Lunch
13.30 - 14.30: Business Meeting of Embryology Special Interest Group

Topic 3 : In vitro culture conditions for early and late embryo development

14.30-15.00: Metabolic requirements – H. Leese (UK)
15.00-15.30: Culture systems for early embryo development – Y. Menezo (FR)
15.30-16.00: Coffee break

Topic 4: Clinical practice: should we transfer at day 2/3 or 5/6?

16.00-16.30: Day 5/6 embryo transfer has no advantage over day 2/3 transfer using the current culture systems – E. Kolibiakis (B)
16.30-17.00 Day 5/6 transfer is the best strategy to reduce the number of embryos transferred in order to avoid multiple pregnancy – D. Gardner (USA)

In vitro maturation of oocytes : basic requirements

Pascal Mermillod
INRA, Unit de Physiologie de la Reproduction et des Comportements
UMR INRA – CNRS
Université de Tours
Nouzilly
France

Learning objectives

- Be able to list the major features of oocyte maturation
- Understand several parameters regulating oocyte intrinsic quality
- Know the environmental requirements of in vitro maturing oocytes
- Be able to list the different ways of future improvements of this technique

Introduction

In domestic mammals, the oocyte is the corner stone of a wide range of biotechnologies. Among these technologies, in vitro embryo production (IVP) through in vitro maturation (IVM) and fertilisation (IVF) of immature oocytes and in vitro development (IVD) of the resulting embryos to the blastocyst stage is commonly used in different species. IVP is useful for the amplification of high genetic merit females, for the safe commercial exchange of genetic gain and for the preservation of genetic diversity (endangered domestic breeds or wild species). High quality oocytes are also required for the set up and use of new technologies such as nuclear transfer cloning or transgenesis. In addition, the oocyte represents a unique model for basic studies on cell cycle regulation and post transcriptional control of gene expression. Therefore, high research potential is devoted to the study of oocyte quality in domestic and model species. Among the domestic species, the bovine receives probably the strongest research efforts and most of our present knowledge of domestic mammals oocyte differentiation and maturation has been drawn from bovine studies and will be presented here. In human, assisted reproductive techniques usually rely on the gonadotropin stimulation of follicular dynamic, leading to the production of several mature oocytes at each IVF cycle. However, the use of IVM may present several advantages over hormonal stimulation of ovarian activity (avoid the use of heavy hormonal treatment of the patient, eliminate the risk of ovarian hyperstimulation and other side effects, improve the results with patients presenting low response to stimulation).

During fertilisation, each gamete brings half of the DNA content of the future embryo. In addition, the oocyte brings most of the zygotic cytoplasm. This cytoplasmic complement appears very important for the success of the development of the embryo since its protein and mRNA content will support all embryo requirements during the genome silencing occurring during the first embryonic cleavages. The quality of the oocyte may be defined as the ability of its cytoplasm to support this critical period of early development. This quality is the cumulated result of the long differentiation process occurring during folliculogenesis and of the profound changes in cytoplasmic structure and content occurring during oocyte maturation, just before ovulation.

Physiological background

Oocyte differentiation
In the ovary, all oocytes are blocked at the prophase stage of the meiotic cycle. The block of meiotic progression during follicular growth is controlled by unknown factors produced by the surrounding somatic cells. At this stage, the round shaped nucleus, called the germinal vesicle (GV), contains diffuse chromatin and is transcriptionnally active. During early folliculogenesis, the oocyte grows, undergo strong morphological modifications and stores molecules (proteins, RNAs) that will be determinant for the success of fertilisation and early embryo development. The zona pellucida, a glycoprotein envelope surrounding the oocyte membrane, is synthesised by the oocyte during this period of growth. The zona pellucida has important function in regulating fertilisation and protection of the embryo until hatching. In physiological conditions, the oocytes resume meiosis and progress to the metaphase II stage only if they encounter a gonadotropin surge in the preovulatory follicle. They stop their progression at this MII

stage until fertilisation in the oviduct, after ovulation. Most of the follicles never reach this stage and are lost during follicular atresia.

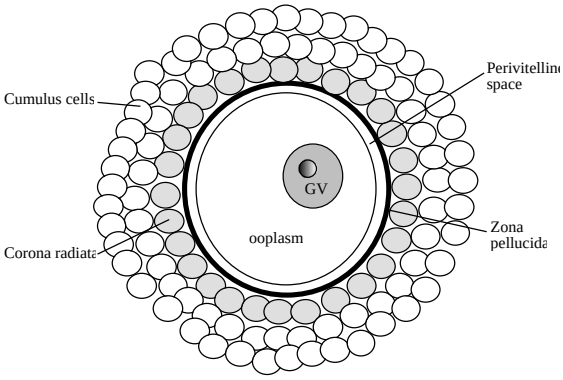

Figure 1 :The cumulus oocyte complex (COC). The germinal vesicle stage oocyte is surrounded by cumulus cells. The cells closer from the zona pellucida form the corona radiata. These cells maintain contact with the oocyte plasma membrane by foot processes crossing the zona pellucida and forming gap junctions. The ooplasm is opaque due to high lipid droplets load. The perivitelline space is virtual in immature oocytes and becomes visible in the mature one, after emission of the first polar body

Oocyte maturation

Immature oocytes that are collected from growing follicles for IVP are blocked at the prophase stage of meiosis These oocytes are surrounded by several layers of cumulus cells (cumulus oocyte complex or COC, Figure 1). These cells communicate together and with the oocyte through a complex network of gap junctions and participate to oocyte differentiation. As soon as oocytes are removed from the follicular inhibitory environment, meiotic resumption occurs spontaneously and progress to metaphase II. This spontaneous meiotic resumption of the oocyte outside of the follicle is the basis of in vitro maturation. In addition to meiotic maturation, the oocyte also undergoes cytoplasmic maturation.

Cytoplasmic maturation covers all morphologic and molecular events accompanying nuclear maturation after LH surge in preovulatory follicles and preparing oocyte cytoplasm to successful fertilisation and embryo development. Cytoplasmic maturation includes well known morphological modifications, such as the migration of cortical granules in the cortical region of the ooplasm. These granules are stored during oocyte growth and release their enzymatic content in the perivitelline space after fertilisation. These enzymes modify the structure of the zona pellucida, preventing the penetration of additional spermatozoa. Meiotic competence is acquired during early folliculogenesis, soon after the apparition of the antral cavity in the follicle. However, after this acquisition, the oocyte requires a further differentiation period during late follicular growth to reach the full competence for cytoplasmic maturation. This late differentiation occurs under the inhibiting signal sent by somatic follicular cells that maintain the meiotically competent oocyte at the prophase stage.

During IVP, more than 90% of the oocytes collected from follicles larger than 3 mm in cattle are able to complete nuclear maturation. However, few of them are competent for cytoplasmic maturation. Consequently, only 30 to 40% of the oocytes reach the blastocyst stage after IVF and IVD (Figure 2). When oocytes harvested from larger follicles or in vivo matured oocytes are processed under the same IVF – IVD techniques, the success rate is increased. This contrast highlights the functional importance of oocyte cytoplasmic quality. In contrast, oocytes from homogeneous origin allotted to different maturation treatments may also provide different development results, showing the importance of the maturation step in addition to immature oocyte quality in determining final oocyte competence.

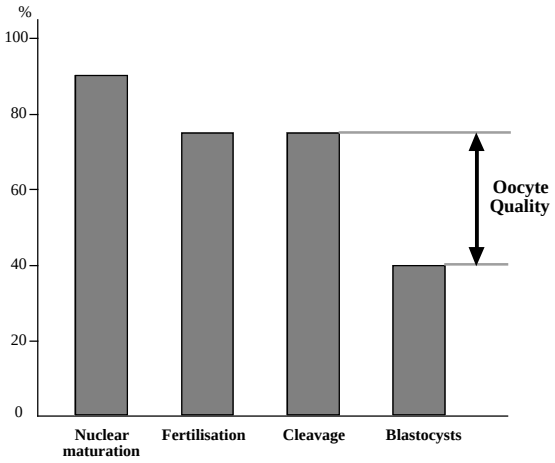

Figure 2: Success rate of the successive IVP steps

Immature oocyte intrinsic quality

The effect of follicle size on oocyte quality evaluated by IVP success rate has been evidenced in numerous species, including human [1-3]. When follicles are dissected and classified according to their size (size classes are different between species), the oocytes harvested from larger follicles provide better development results than those from smaller follicles. Consequently, large follicles contain a higher proportion of developmentally competent oocytes. Since we talk about Ç blastocyst rate È at the end of IVP and that some blastocysts are observed even when oocytes are harvested from smaller follicles, it is more likely that oocyte capacitation is occurring with different kinetics between follicles. If we consider the smaller follicles, only a few oocytes appear competent (the faster oocytes) whereas in larger follicles, even slower developing oocytes have had time to complete capacitation.

Several factors may influence this kinetic of oocyte differentiation. For example, a functional disjunction between follicular growth and acquisition of meiotic and developmental ability by the oocyte was found in ewes bearing the booroola fecundity mutation. It has been shown that in heterozygous ewes (Fec^{B} Fec^{+}), the rate of development of oocytes from a given follicular size class was superior to the rate observed for oocytes obtained from the same size class in females of wild (Fec^{+} Fec^{+}) genotype [4]. As a consequence, a single gene may be able to regulate the kinetics of differentiation of the oocyte inside of the growing follicle. The booroola mutation has been identified as an inactivating single nucleotide mutation of the BMP receptor 1B gene. The bone morphogenetic proteins, members of the transforming growth factor b family (*BMP15, GDF-9*, BMP receptors) have been recently evidenced as central players of the oocyte – somatic cells communication responsible for the coordinated differentiation of somatic and germinal compartments of the ovarian follicle [5]. Other factors are known to influence the kinetic of oocyte differentiation during follicular growth (genetic background of the female, localisation of the follicle, puberty of the female, follicular atresia).

The morphological and biochemical basis of oocyte competence remain to be determined, however, it probably involves the transcription of some genes important for survival or for the regulation of gene expression in early embryos. Recent development of molecular tools allowed to increase our knowledge of oocyte gene expression [3, 6] and leaded to the identification of several maternal effect genes (*mater, zar-1, npm2, Dnmt-1o,* É). These genes are more or less specifically expressed in oocytes and their expression is essential for the success of early development, as evidenced by the impaired development beyond the 2-Cell stage observed in knock out female mice. An oocyte specific homolog of *mater* gene has been found recently in human [7], opening the way for a better understanding of molecular basis of oocyte competence in this specie.

In vitro maturation treatment

In vitro maturation is probably the most critical part of the whole process of in vitro embryo production. Ruminant oocytes are usually matured at 39¡C under a 5% CO_2 in a humidified atmosphere. The optimal maturation time (more than 90% of the oocytes at the metaphase II stage) is 22-24h. Due to the high lipid content of oocytes cytoplasm, it is not possible to follow the progression of their nuclear status during culture. Additionally, the presence of the cumulus cells is required during IVM for efficient cytoplasmic maturation and these cells mask the oocyte (Figure 3). Consequently, the only visible sign of oocyte maturation during IVM is the expansion of the cumulus cells. These

cells produce hyaluronic acid which is secreted and polymerised in the extracellular matrix, leading to the increase of intercellular space (Figure 3).

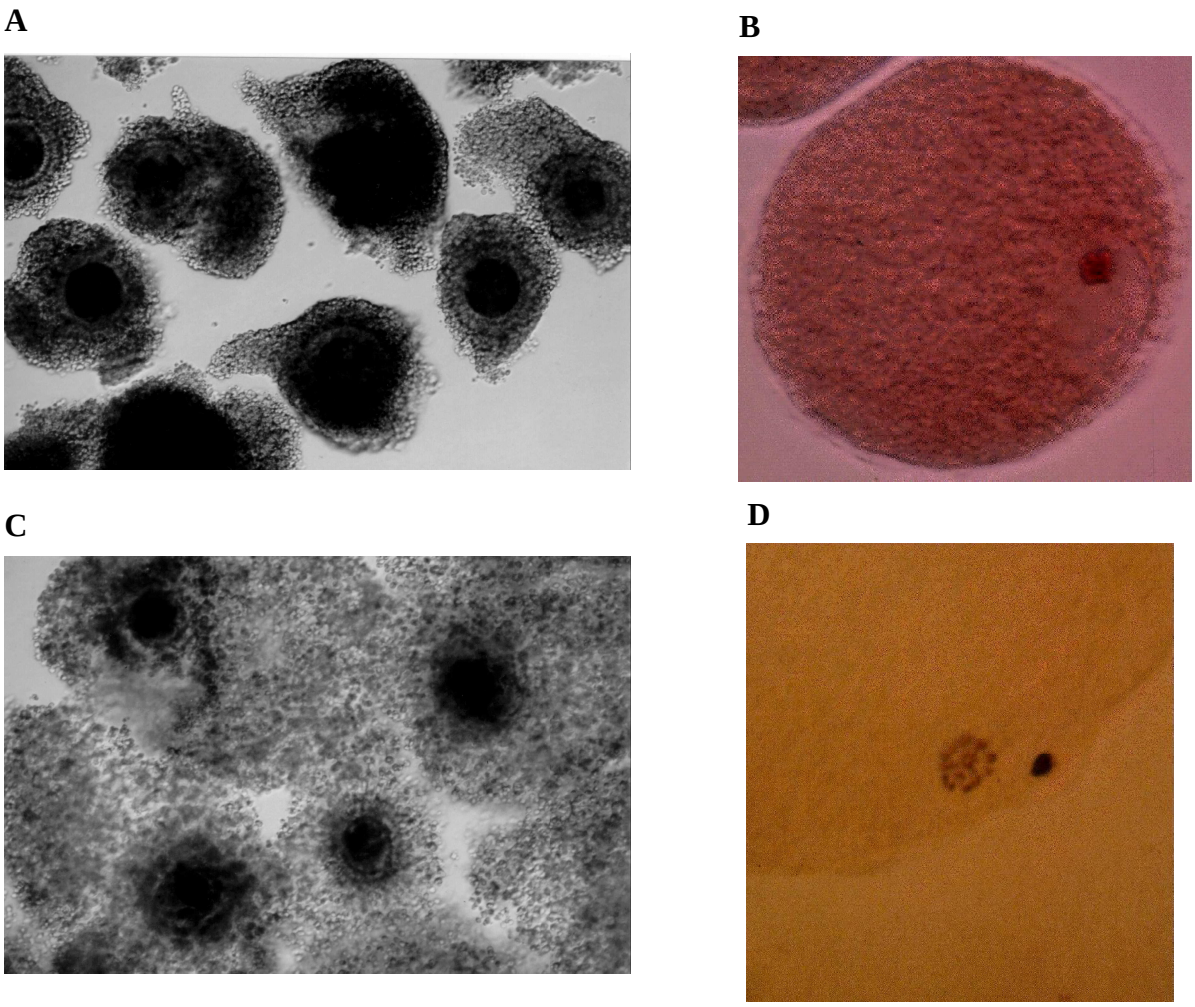

Figure 3 :Bovine cumulus oocyte complexes at the time of collection, the cumulus investment makes difficult to see the oocytes, the corona radiata and zona pellucida are visible in some of the COC (g=120x). (B) Orcein stained immature oocyte, the cumulus cells have been removed to allow to see the germinal vesicle with a large nucleolus (g=600x). (C) Bovine COC after in vitro maturation, note the expansion of the cumulus cells (g=120x).
(D) Orcein stained bovine oocyte after in vitro maturation, note the metaphase II plate and the extruded polar body (g=1000x).

Several culture media have been proposed for IVM (MEM, Waymouth, Ham-F12). However, the most efficient one seems to be the TCM199 medium, bicarbonate buffered and containing minerals, carbon and energy sources (glucose, glutamine) as well as vitamins and amino acids. The medium is generally supplemented with high molecular weight molecules that exert a surfactant effect (BSA) and provide hormones and growth factors (foetal calf serum, serum of female in oestrous, follicular fluid). These complex additives from animal sources may raise sanitary questions due to possible presence of pathogen agents, and decrease the reproducibility of experiments. They may be successfully replaced by high molecular weight polymers for the surfactant effect (such as polyvinyl alcohol) and by cocktails of purified or recombinant hormones and growth factors.

Hormones are usually added to the maturation medium (FSH, LH, estradiol), in addition to biological fluids, although their exact function is not clearly established. Growth hormone (GH) stimulates some aspects of cytoplasmic maturation (cortical granules migration) as well as nuclear maturation, resulting in a higher blastocyst yield. GH action seems to be mediated by cumulus cells. Epidermal growth factor (EGF) has been shown to stimulate nuclear and cytoplasmic maturation in a wide variety of species including human, pig, cattle and sheep. High blastocysts yields could be obtained after maturation of cattle oocytes in TCM199 only supplemented by EGF. EGF action is exerted through

cumulus cells as well as directly on the oocyte. Insulin like growth factor-I (IGF-I) has also a positive effect on cytoplasmic maturation of cattle oocytes. In ruminant species, maturation lasts 24 h whereas 44 h are required for complete maturation of porcine and human oocytes.

Recent data using specific inhibitors of the M-phase Promoting Factor (MPF, key regulator of oocyte meiotic resumption) suggest that oocytes may be reversibly maintained in vitro at the germinal vesicle stage [8]. Such treatments may allow in a near future the establishment of two-steps culture systems including a pre maturation culture under meiotic inhibition to increase the proportion of competent oocytes before maturation.

One of the most characteristic feature of oocyte maturation in culture is the parallel differentiation of cumulus cells. This differentiation translates into cumulus expansion due to production of a mucified extracellular matrix. In addition, cumulus cells also display changes in their gene expression patterns, including the activation of the expression of some genes, some of them being regulated by oocyte originated factors (cyclooxygenase-2 for example). Some of these maturation related cumulus expressed genes may represent predictive markers of the competence of a given oocyte.

Conclusions

Both immature oocyte intrinsic quality and conditions used for in vitro maturation determine the final developmental competence of the oocyte entering the fertilisation process. A better knowledge of oocyte differentiation process will help the identification of molecular markers of immature oocyte quality (in oocytes themselves or in surrounding somatic cells) which in turn will facilitate the selection of oocytes and the design of hormonal treatments to improve the quality obtained from one given female. The increasing knowledge of the regulation of oocyte cell cycle regulation will allow designing of culture systems that will permit to immature oocytes to reach full competence before maturation. Finally, the better knowledge of growth factors effects and requirement during oocyte maturation will lead to the definition of optimised in vitro environment for a better success of in vitro maturation.

References

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In vitro maturation of oocytes. Clinical experience.

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Herlev
Denmark

Introduction (fig 1).

The basis of IVM is the maturing in vitro of oocytes from Germinal Vesicle (GV) stage of development to the metaphase II stage. Oocytes are retrieved for IVM from antral follicles 2-10 mm diameter and matured in vitro for 24 to 52 hours. In fully grown oocytes resumption of meiosis in vivo is triggered by lutheinizing hormone (LH) surge. Removal of the oocyte from the follilcle is the corresponding in vitro signal.

Maturation and developmental competence of the oocyte (fig 2 – 3).

Oocyte maturation includes nuclear and cytoplasmic events. The end point whether in vivo or in vitro is a methaphase II (MII) oocyte which can be fertilized and which can support normal embryonic development. Nuclear maturation is characterized by breakdown of the nuclear membrane, separation of homologous chromosomes and extrusion of the first polar body into the perivitelline space. Beyond these nuclear aspects of oocyte maturation cytoplasmic events occur and they seem to be important for fertilization and developmental ability of the oocyte.These aspects have been termed cytoplasmic maturation.

Nuclear and cytoplasmatic maturation is normally highly co-ordinated during normal reproductive cycles, but this co-ordination can be uncoupled by in vitro maturation technologies. Most deficiencies in oocytes during in vitro maturation are associated with cytoplasmic reprogramming rather than meiotic progression, for reasons, which remain unknown.The effects of cytoplasmic aberrations are seldom expressed at an early stage of development but instead more frequently associated with cleavage and peri-implantation stages. Therefore, fertilization and pronuclear development may occur in a wide spectrum of eggs, which lack the capacity to develop normally to term and the guide to the full developmental capacity is delivery of healthy infants.

IVM technique (fig 4).

Aspiration
Ultrasound guided transvaginal aspiration of immature oocytes was first described by Trounson and co-workers, 1994. They introduced two major modifications compared to conventional IVF ultrasound guided oocyte pick-up. Firstly, a new more rigid aspiration needle with a shorter bevel at the tip (Cook, Australia Ltd.) and secondly, a reduced vacuum of 80 - 100 mm Hg. The reduced vacuum seemed to be far the most significant change. The adapted Cook needle and standard double lumen needle may be used with no difference in recovery rate. Most reports have described the use of a single lumen needle under ultrasound guidance and follicles of 2-10 mm may be aspirated without flushing. L.`

In previous studies transvaginal oocyte aspiration has been performed under general anaesthesia or spinal anaesthesia. Russell *et al.* described the use of paracervical block.

Culture medium
Very few reports based on human data are available on the composition of culture media for human oocyte maturation. Furtherrmore, often too few GV oocytes have been available for stating significant comparisons. Improvements in human oocyte maturation and embryo cleaving in the presence of FSH and LH have been reported. However, there is still a need for considering the variations in relative concentrations for optimizing developmental capacity.

Culture medium for human IVM is usually supplemented with serum. The most commonly used protein sources in human IVM are fetal cord serum and fetal bovine serum. Due to potential sources of infectious agents it has been

advised not to supplement with serum sources from other patients or from animals, and therefore the patient's serum has been used. Some clinics have used HSA or synthetic serum substitute as protein supplementation in IVM. HSA is a relative pure fraction, although its content can also be variable. The serum concentrations have varied between 7.5% and 20% and the concentration of HSA and synthetic serum protein has varied between 0.1% to 0.4% and 10%, respectively. Significantly increased rates of maturation, pregnancy and implantation have been obtained from oocytes matured in culture medium with serum supplementation compared to oocytes matured in medium supplemented with HSA. The reasons for the higher performance of serum supplemented media in the IVM system remain to be elucidated.

Time interval of maturation.
Previous studies have shown, that 80% of immature human oocytes show nuclear maturation (extrusion of a polar body) and will be at MII by 48-54 hours of culture. A considerable asynchrony of the maturation has been observed and a number of MII oocytes can be obtained already after 24 hours of maturation. If these oocytes are inseminated after 48 hours they have been at MII arrest for 20-30 hours, which places them well past the optimal fertilization time and may compromise their developmental competence.

No significant difference in rates of maturation,fertilization or pregnancy, was observed, when oocytes were matured for 28 hours compared to 36 hours. The optimal time of insemination has not yet been established. The 28-hour IVM period had a significant benefit in that it allowed the insemination to be performed during working hours; it had to be performed at night when the 36-hour IVM schedule was used.

Fertilization, embryo culture and priming of the endometrium.
When comparing to conventional insemination technique, ICSI results in higher fertilization in human oocytes matured in vitro. A fertililzation rate of 45% was reported, when oocytes were inseminated conventionally compared to 70-75% when ICSI was performed Furthermore, by removal of granulosa cells it was possible to identify the extrusion of the first polar body, i.e. a MII ova.

Puncturing follicles before they have reached maturity may result in an endometrium that is inadequately primed for implantation due to lack of adequate endogenous oestradiol and progesterone produced by granulosa cells. Exogenous priming with oestradiol and progesterone is needed and one must synchronize the window of implantation with the embryo development. It is well known from hormone replacement in recipients of donor oocytes that 2-day-old embryos are best transferred into the endometrial cavity on day 3 or 4 of progesterone exposure.Imitating the normal priming as closely as possible with initiating oestradiol on the day of aspiration and supplemented with progesterone 2 days later has been suggested.

Selection of patients for IVM (fig 5 - 7).

1.Immature oocytes from natural cycles.
Optimizing of IVM could begin with appropriate selection criteria to determine which patients could benefit from this tool. In terms of the predictors of success for IVM in regularly cycling women with normal ovaries one criterion appeared to be a low basal level on day 3 of oestradiol (< 200 pmol/l). The concentration of FSH and the number of follicles on day 3 predicted the number of oocytes retrieved, whereas these parameters did not predict the subsequent development of oocytes.

Few prospective studies have examined the effect of priming with FSH before aspiration of immature oocytes in regularly menstruating women. The series are small, a variety of stimulation regimens have been used. Similar rates of maturation, fertilization, cleavage and pregnancy were observed, if oocytes were obtained after a follicle of 10 mm was observed by ultrasound. FSH priming had to be followed by deprivation for 2-3 days. This observation is consistent with observations in bovine studies where competent oocytes should originate from early atretic follicles. In unprimed natural cycles the oocytes have been retrieved at different moments in the menstrual cycle and probably from follicles of different sizes. The recruitment and growth of numerous follicles characterize each menstrual cycle. One or two selected follicles continue to grow until the day of ovulation, while the remaining follicles untergo atresia. We aimed at oocyte collection to coincide with selection of the dominant follicle. Oocytes were aspirated after a leading follicle of 10 mm and an endometrial thickness of at least 5 mm were observed at ultrasound and in 87 cycles a pregnancy rate of 12.6% per aspiration was obtained. Serum levels of oestradiol and inhibin A were evaluated retrospectively. Significantly more oocytes were obtained in cycles with a detected increase in the level of oestradiol from day 3 to the day of aspiration (19% per aspiration) compared to cycles without such an increase. A higher pregnancy rate was observed after an increase in the level of inhibin A concentration (24% versus 0%) per aspiration.

Oocytes originating from the ipsilateral ovary did not show to have an impaired competence to mature and cleave compared to oocytes originating from the contralateral ovary.

Immature oocytes from PCOS patients.
Although pregnancy rates of 27% have been reported, the implantation rate has been low (6.9%) for embryos obtained after IVM on immature oocytes from unstimulated PCO patients. Rates of maturation, fertilization and cleavage between untreated regular ovulation and irregularly or anovulatory polycystic women have been examined and in almost all the parameters analysed oocytes from regularly cycling patients performed better. The reason for this was not determined. To compensate for this, endogenous hCG has been shown to improve the maturation rate of immature oocytes in PCOS patients. Furthermore priming with rec-FSH followed by coasting for 2-3 days before aspiration of immature oocytes may improve the maturational potential of the oocytes. In a prospective randomized study an implantation rate of 21,6% was obtained in the FSH primed group compared to 0% in the non-primed group.

Safety of the IVM procedure and effects on offspring (fig 8).

In our center 42 children have been born. The median weight was 3770 gram (range 1745 gram to 4690 gram). One girl had a soft cleft palate, the remaining had no malformations and they are all healthy, the oldest child being 5 years old. The first 18 babies have been followed-up by examination when they are 6 months, 1 year and 2 years old.

Future perspectives (fig 9).

The data taken together suggest that in future immature oocyte retrieval combined with IVM could possibly replace standard stimulated IVF in selected patients.IVM is easy, cheap and the risk of ovarian hyperstimulation is eliminated. Through further research it may be possible to refine and optimize the conditions for IVM.

In the future in vitro follicle culture in combination with IVM may have advantages in assisted reproductive technologies and may help to restore fertility in the treatment of cancer in children and young women. Research is continuing in optimizing the methods for the freezing of isolated immature oocytes and of a complete human ovary, and progress is being made. not yet been reported. While cryopreservation of reproductive cells and tissue is already becoming established, in vivo preservation will be a key goal in the future when the implications of inhibiting apoptosis or oocyte quality are better understood. Research is continuing in optimizing the methods for the freezing of isolated immature oocytes and of a complete human ovary. This may have advantages in assisted reproductive technologies and may help to restore fertility in the treatment of cancer in children and young women.

References.

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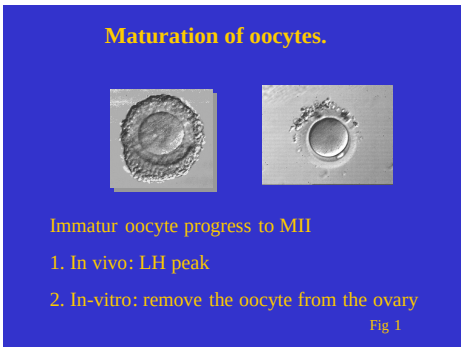


Nuclear maturation

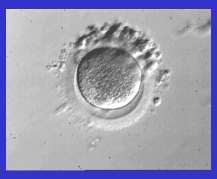
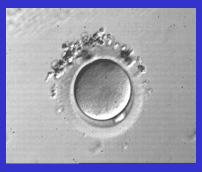
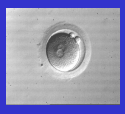
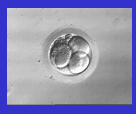



Fig 2

Cytoplasmic maturation

Processes that prepare the oocyte for fertilization and subsequent embryo development

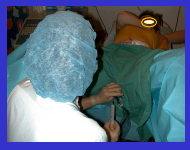



No markers – measured indirectly by

pregnancy rate - full term live born baby.

Fig 3

IVM - technique



Maturation:	28 - 36 hours
Fertilization:	ICSI
Endometrial priming:	oestradiol per os and intravaginal progesterone

Fig 4

IVM
regularly cycling women

Predictive factors on day 3:
– the level of oestradiol, inhibin A

In vivo FSH priming:
– no impact

Timing of aspiration in unstimulated cycles:
– Aspiration the day after a follicle of 10 mm

Fig 5

IVM - Timing aspiration Unstimulated cycles

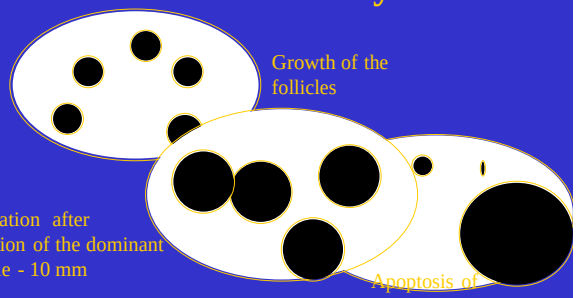


Fig 6

IVM

Fig 7

IVM total cohort

- 24 boys and 18 girls
- 2 preterm deliveries
  - Severe preeclampsia : gestational age 32 weeks
  - Twins: gestational age: 34 weeks
- 38 deliveries at term
  - gestational age 40 weeks (37 - 42 weeks)
  - median weight 3770 gram (range 3000 - 5260 gram)

Fig 8

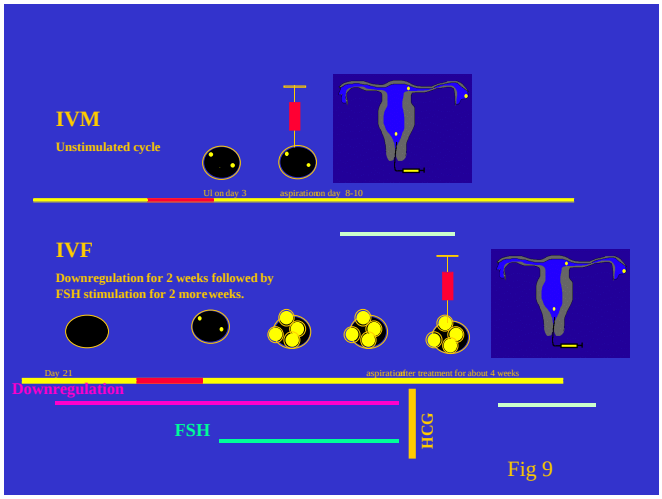


Fig 9

In vitro maturation of male germ cells: which end points are required ?

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Learning objectives

Many attempts have been performed over the last decade to create the *in vitro* conditions propitious to male germ cell proliferation and differentiation. In the human species the ethical possible experimental protocols may be limited. By contrast, animal models allow to define appropriate methodologies and criteria of evaluation in order to get proofs of the *in vitro* development of male gametes and to assess their normality.

Introduction

During spermatogenesis, diploid spermatogonia divide mitotically several times to provide a population of spermatocytes that proceed through meiosis to give birth to haploid spermatids; these latter undergo, during spermiogenesis, a morphological differentiation leading to spermatozoa. Spermatogenesis takes place in the seminiferous epithelium where the germ cells are in close association with the Sertoli cells. Multiplication, differentiation and survival/apoptosis of germ cells, are finely regulated by hormones, mainly by FSH, acting on Sertoli cells, and LH, through the production of testosterone by Leydig cells, and a multitude of cell-cell interactions involving membrane bound or secreted factors such as growth factors, cytokines and neurotrophins. It is becoming clearer and clearer, that hormones and intratesticular regulatory factors may compensate, at least in part, the absence of some of them including FSH and androgen or LH receptors. Thus, it is likely that synergism and/or redundancy between regulatory molecules is a characteristic of the spermatogenic process on which depends species survival.

Many teams have studied which regulating factors are produced within the testis. However, for many of these factors, their specific action on germ cell differentiation is unknown. This search has been hampered by the lack of long-term culture systems creating the *in vitro* conditions necessary for male germ cell development. Indeed, such culture systems should be helpful in establishing the role of paracrine factors, either by adding them to the culture medium, or by preventing their expression or blocking their action *in vitro* with use of oligonucleotides or specific antibodies. Such studies should be important to identify some of the causes of male infertility. Moreover, *in vitro* maturation of male germ cells would allow to preserve the potential of reproduction of children treated with chemotherapy or radiotherapy, and could allow to cure some infertility, due to defects in somatic cells of the testis, by culturing germ cells in a suitable environment for their maturation. Moreover, germ cells (spermatogonia) might be used as cellular vectors for transgenesis in domestic animals. Hence several groups, during the last years, have tried to settle culture systems allowing some steps of multiplication/differentiation of mammalian germ cells to occur *in vitro* in several species: rat, mouse, pig, bull, and also in the Human. However, several parameters should be evaluated in order to ascertain the functionality of these culture systems and the normality of the *in vitro* differentiated gametes. For better clarity, and because of space limitation, this short presentation will focus on the more recent studies. References of earlier works may be found in the referenced recent papers.

***In vitro* multiplication and differentiation of spermatogonia**

The mitotic phase of spermatogenesis is a complex series of events involving likely differentially regulated specific factors. The spermatogonial stem cells undergo self-renewal and simultaneously produce more differentiated stages of spermatogonia. In non-primate mammals, the As (single) spermatogonia are considered to be the stem cells of spermatogenesis. Upon division, they produce daughter cells, which either become new single stem cells or remain connected through an intercellular bridge giving A-paired (Apr) spermatogonia. These latters develop further into chains of A-aligned (Aal) spermatogonia. The Aal spermatogonia differentiate into A1 spermatogonia and, after

several mitotic divisions into A2, A3, A4, A intermediate spermatogonia, give birth to B spermatogonia. The B spermatogonia will give rise to preleptotene spermatocytes at the ultimate meiotic division. Hence, the undifferentiated spermatogonia include As, Apr and Aal spermatogonia, whereas the differentiating spermatogonia include the A1, A2, A3, A4, intermediate and B spermatogonia.

Many attempts to settle culture systems allowing proliferation of spermatogonial stem cells and differentiation into meiotic spermatocytes have been reported during the last decade. The earlier studies were based on light microscopy, identification of germ cells, and labelling S phases with ^{3}H-thymidine or Bromo-2'-deoxyuridine (BrdU). Since morphological differences between undifferentiated spermatogonia and A1-A2 spermatogonia are quite small in the rat, it is very difficult to distinguish the undifferentiated spermatogonia from the A1 and A2 types of differentiating spermatogonia, especially in cultures. Hence, in the second generation of experiments, specific markers for the different types of spermatogonia and for somatic cells were used in immunocytochemical experiments, and often associated to electron microscopy. Moreover, the evaluation of stem cell activity of cultured spermatogonia has been evaluated by transplantation of germ cells in the testes of infertile or nude mice, in the case of heterospecific transplantations. These latter experiments have allowed to identify specific markers of spermatogonial stem cells.

Development of the meiotic step *in vitro*

Meiosis is a unique event, which is restricted to germ cells. It includes the pairing and recombination of chromosomes during prophase of meiosis I, and the segregation of homologous chromosomes during anaphase of meiosis I.

After the last mitotic division, diploid spermatogonia develop into preleptotene spermatocytes which become 4C cells after premeiotic DNA replication. The first meiotic division then generates haploid secondary spermatocytes, which possess two copies of each gene (2C cells). After the second meiotic division, the secondary spermatocytes generate haploid spermatids, which will differentiate into spermatozoa. Recently a part, then the whole meiotic stage of spermatogenesis was reproduced *in vitro* in different species. The meiotic process was monitored by four criteria: i) morphological identifications of newly formed spermatids from seeded spermatocytes, by both light and electron microscopy ; ii) determination of the change in ploidy of the cell population seeded with time in culture ; iii) assessment of the ability of germinal cells to transcribe genes expressed during the post meiotic phase ; iv) monitoring the fate of BrdU-labeled preleptotene spermatocytes over the culture period until the identification of BrdU-labeled round spermatids. A recent study aimed to compare some features of the meiotic process, which develops in the testis of pubertal rats, *in vivo* and *in vitro*, paying special attention to the time-course of the phenomenon. The differentiation of spermatocytes was assessed in testes of 20- to 46-day-old rats and in tubule segments of 20- or 28-day-old rats cultured over a 4-week period. Very similar results were obtained *in vivo* and *in vitro*, during the first week of culture, when considering the changes in the cell populations of different ploidy, the gene expression of germ cells, the kinetics of differentiation of BrdU-labeled spermatocytes and the levels of apoptosis in the different cell populations. However, during the second week of culture, the decrease in the proportion of the 4C cell population was not associated with an increase in the 1C cell population as large as *in vivo*. This result could be explained partly by a high proportion of apoptotic 1C cells beyond one week of culture. Concomitantly, the rate of *in vitro* differentiation of BrdU-labeled spermatocytes slowed down when reaching the stage of middle pachytene spermatocytes and BrdU-labeled round spermatids were observed 6-11 days later than when BrdU-labeled spermatocytes differentiated *in vivo*.

These results therefore identified a bottleneck for the development of the rat meiotic cells *in vivo* at the transition from middle to late pachytene spermatocytes. These experiments also allowed to make a rough comparison between the efficiency of the meiosis event, which occurs in vivo, and in cultured seminiferous tubules from 20-day-old rats. Indeed, by comparing the number of round spermatids obtained to the number of leptotene spermatocytes seeded, it could be estimated that one leptotene spermatocyte gave birth to about 0.2 round spermatid which is 4-6 fold lower than *in vivo* at 35 days. It has to be noted that in most instances, however, many abnormalities of *in vitro* formed round spermatids were observed. This includes incomplete or delayed nuclear condensation, cytoplasmic elongation, acrosome formation or development of several flagella. Nevertheless, it has been reported very recently that round spermatids developed *in vitro* from spermatocytes can produce normal mouse when injected into mature oocytes, but with a rather low yield (see below).

***In vitro* spermiogenesis**

Actually, there is very few recent data on *in vitro* spermiogenesis in animal models. *In vitro* formed round spermatids can generate one to several flagella, all emerging from the same cellular pole. Moreover, in some instances, actively

propagating bending waves have been reported. However, it is important to emphasize that the presence of flagellated cells in germ cell Sertoli cell coculture systems does not ascertain that these cells are spermatids, since any cell with a centriole-derived basal body is able to generate an axoneme. The composition of the intratubular fluid in which are released mature spermatozoa is very different from that of the extracellular medium. Hence, it is likely that performing the whole spermiogenic process *in vitro* will need a specific culture medium different from those used for germ cell multiplication and meiosis.

Evaluation of the *in vitro* differentiated gamete quality

There is increasing evidence that *in vitro* culture of preimplantation embryos can be associated with aberrant growth and phenotypic abnormalities during fetal and postnatal development. Among the several hypotheses proposed to explain these results, is that *in vitro* culture leads to aberrant epigenetic modifications in the genome.
Genome imprinting results in non-equivalent expression of the paternal and maternal alleles of certain genes. The nature of the imprint that marks the parental alleles is not fully understood. However, it must be capable of being erased and then reset sometimes during gametogenesis, so that oocytes carry the maternal imprint and spermatozoa carry the paternal imprint. It has been shown that the expression of imprinted genes in mouse embryos obtained by round spermatid injection into oocytes do not differ from controls. This strongly suggests that paternal genes are correctly imprinted by the round spermatid stage. However, as mentioned above, the ability of *in vitro* formed round spermatids to give birth to live pups is rather low. Thus it appears important now to compare the profile of gene expression and the imprinting status of *in vitro* and *in vivo* differentiated gametes.

Conclusions

Considerable advances for achieving spermatogenesis in *vitro* have been made during the last decade. An intriguing feature is that it appears that the culture conditions may vary greatly between species and even between different genetic backgrounds in the same species. Hence it appears necessary to identify specific factors which govern the balance between germ cell survival, progression and attrition in the different species to achieve more efficient and normal spermatogenesis *in vitro*.

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In vitro maturation of male germ cells: clinical experience

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Learning objectives

1. Germ cell recognition and staging
2. Clinical indications for the use of in vitro matured germ cells for assisted reproduction
   a) Postmeiotic maturation arrest
   b) Meiotic maturation arrest (use of cultured spermatids after transmeiotic in-vitro differentiation
   c) Sperm DNA damage
3. Improvement of germ cell quality by in vitro maturation
4. Concerns about the use of in vitro matured germ cells for assisted reproduction
   a) Indication
   b) Success rate
   c) Safety
5. Conclusion

1. Germ cell recognition and staging

Traditionally, individual stages of human male germ cells are recognized in histological preparations of testicular biopsy samples. However, the application of assisted reproduction treatment (ART) techniques using immature germ cells for fertilization requires recognition and staging of living and unstained germ cells released from the seminiferous tubules after testicular biopsy. Basic features of individual stages of germ cells in this context have been described (1, 2). These descriptions are based on the size, shape, and chromatin pattern of nuclei, the presence of meiotic metaphase figures (chromosomes), acrosomic vesicles or cap, and tails (flagella), and/or the presence of mitochondria in the middle piece of the tail of germ cells. Briefly, the characteristics allowing the identification of different germ cell stages were the following. Spermatogonia: nuclear size, very distinct nuclear envelope, and chromatin pattern. Preleptotene primary spermatocytes: the smallest nuclei of primary spermatocytes, the finest chromatin clumps, and the least defined nuclear envelope. Zygotene primary spermatocytes: slightly longer nuclei and an enlargement of the chromatin clumps. Pachytene primary spermatocytes: the largest of all germ cells in the testis, one or more large spherical nucleoli. Secondary spermatocytes: fine chromatin and an indistinct nuclear envelope, nuclear size intermediate between primary spermatocytes and round spermatids. Sa spermatids: sometimes a spherical or flattened acrosomic vesicle, fine chromatin, and two or more nuclear clumps. Sb_1 spermatid: an apparent acrosomal cap and a nucleus that had begun to lose its spherical shape. Sb_2 spermatids: elongating nuclei, an annulus located in proximal position of the developing tail (near the head). Sc spermatids: an annulus around the developing tail located in the proximal position (near its head) or distal position (at the bottom of the middle piece of the tail). Sd_1 spermatids (elongating): shortened nuclear head length and a migrated annulus of its developing tail. Sd_2 spermatids (elongated): mitochondria around the middle piece of the tail, a developing cytoplasmic droplet located in the proximal position (next to the head) of the tail.

2. Clinical indications for the use of in vitro matured germ cells in assisted reproduction

a) Postmeiotic maturation arrest

Spermatogenesis can be arrested by a pathological process in the testis at any stage, but premeiotic and meiotic blocks represent the most frequent situations (3). However, postmeiotic arrest of spermatogenesis does occur in some patients, and this was the original indication for the use of spermatids in assisted reproduction, leading to the first pregnancies and births after fertilization with round (4) and elongated (5) spermatids.

b) Meiotic maturation arrest

Recent work with testicular biopsy samples from men with obstructive azoospermia (relatively intact spermatogenesis) has shown that, under appropriate conditions and in the presence of high concentrations of FSH (>25 IU/l) and testosterone (l mM), a subpopulation of germ cells undergo a very rapid (1-2 days) meiotic and postmeiotic differentiation during in-vitro culture (6). When the same methodology was applied to 5 men with maturation arrest at the primary spermatocyte stage, the in-vivo arrested spermatogenesis was resumed in 2 of them and a few postmeiotic germ cells, including morphologically atypical elongated spermatids in one case, were detected after 2 days of culture (7). In both cases, oocytes microinjected with the in-vitro developed spermatids were fertilized and underwent early cleavage divisions, and a twin pregnancy, resulting in the birth of normal babies, was established in one of them (7). Another live birth with in vitro matured germ cells from a man with spermatogenic arrest at the primary spermatocyte stage has been reported recently (8).

c) Sperm DNA damage

In vitro maturation of male germ cells has been proposed for assisted reproduction treatment also in cases in which spermatogenesis is not completely arrested but a high proportion of postmeiotic germ cells shows signs of sperm DNA damage (9, 10). After an initial case report (9) a subsequent pilot study reported live births in a series of cases with previous repeated failures of assisted reproduction (10).

3. Improvement of germ cell quality by in-vitro maturation

Clinical studies using in vitro matured germ cells for assisted reproduction made use of a culture system in which germ cells were incubated in segments of explanted seminiferous tubules maintaining the original association with Sertoli cells (6).

In addition to making it possible to overcome the in-vivo meiotic block and to obtain a few spermatids for assisted reproduction in some patients with maturation arrest at the primary spermatocyte stage (7), in-vitro culture can bring about further benefits. First of all, in vitro matured germ cells undergo additional nuclear and cytoplasmic maturational changes which may be beneficial for their reproductive capacity (11). Moreover, the frequency of apoptotic germ cells is significantly lower in cultured aliquots of testicular biopsy samples as compared to freshly recovered aliquots originating from the same samples (12, 13).

Recently, the possibility of overcoming maturation arrest at the primary spermatocyte stage has been confirmed with the use of a different in vitro culture system using co-culture of isolated germ cells on Vero cell monolayers (14). Reproductive capacity of spermatids formed with this culture system remains to be assessed.

4. Concerns about the use of in vitro matured germ cells for assisted reproduction

a) Indication

Some years ago, the clinical interest in the use of spermatids for assisted reproduction was a debated issue since several workers claimed the inexistence of postmeiotic maturation arrest. However, recent work by several independent groups shows clearly that spermatogenesis can indeed be arrested both at the round spermatid stage and in the course of spermatid elongation process even though these types of maturation arrest are less frequent as compared to maturation arrest at meiotic stages, namely at the primary spermatocyte stage (reviewed in 15). Moreover, the use of in-vitro differentiated spermatids is the desired endpoint of culture techniques aimed at overcoming the in-vivo maturation arrest at earlier stages of spermatogenesis, and at obtaining healthy gametes in men with massive sperm DNA

damage (see above). A lack of therapeutic indications is thus certainly not an argument against further development of germ cell in vitro maturation techniques.

b) Success rate

The ongoing pregnancy rate of assisted reproduction with in vitro matured germ cells still remains low, especially when only round spermatids are available after culture. In cases of postmeiotic (spermatid) arrest the current success rates are 5-30%, depending on the blocking stage (round, elongating or elongated spermatid) and the female age. For meiotic arrest (primary spermatocyte) ongoing pregnancies have been achieved only exceptionally (3 babies born reported in the literature). In these cases the patient's decision to have recourse to these techniques will be conditioned by the value that the couple attaches to the possibility of having progeny of their own genetic constitution as well as by geographical, cultural, legal and administrative factors which affect the availability of alternative solutions, such as the use of donor sperm or adoption.

On the other hand, much higher success rates have been reported when in vitro maturation is used to promote selection of healthy gametes in cases of complete spermatogenesis with massive sperm DNA damage (10).

c) Safety

Most concerns about the use of in vitro matured germ cells for assisted reproduction were related to the risk of chromosomal abnormalities of the resulting embryos, apoptotic DNA damage in developmentally blocked germ cells, incompleteness of genomic imprinting and the risk of transmititng abnormal mitochondrial genomes.

The risk of chromosomal abnormalities is mainly associated with the use of in-vitro cultured germ cells that have undergone the final phase of their meiotic divisions in culture. The use of intra-oocyte injection of primary spermatocytes into metaphase II oocytes in order to achieve haploidization was indeed burdened by a high frequency of chromosomal errors, namely premature sister chromatid segregation (16). In contrast, preliminary obervations on human spermatids resulting from in-vitro transmeiotic differentiation did not reveal any dramatic increase in the frequency of aneuploidy, and the two babies born after the clinical application of this technique had normal karyotypes (7). The risk of choosing a spermatid with apoptotic DNA damage for assisted reproduction is decreased by the use of in-vitro culture, leading to selection of non-apoptotic cells (12).

7. Conclusions

Male germ cell development may be arrested by different kinds of testiculopathy at any stage of spermatogenesis including spermiogenesis. The use of spermatids at different stages of postmeiotic differentiation for assisted reproduction in cases of spermiogenesis arrest has been reported by different centres. In addition, in-vitro developed spermatids have been used in cases of meiotic maturation arrest at the primary spermatocyte stage. Success rates of assisted reproduction with spermatids are higher when elongating or elongated spermatids (developing in vivo or resulting from in-vitro culture) are available. In contrast, success rates are low when round spermatids are used, mainly because of a high risk of inadvertent use of spermatids carrying apoptosis-related DNA damage. Some improvement has been achieved by the use of in-vitro cultured spermatids for assisted reproduction instead of freshly recovered ones. In vitro maturation culture system has recently been used with success in cases of male infertility due to sperm DNA damage.

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In vitro culture conditions for fertilization. Capacitating media - Basic requirements

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Learning objectives:

1) To define capacitation.
2) To describe several key elements of capacitation.
3) To outline difficulties in precisely defining capacitation.
4) To identify components of in vitro culture media that promotes or hinders capacitation.

Introduction

Capacitation can be characterized as structural and functional changes in the spermatozoon that: 1) become initiated after the removal of stabilizing proteins resident in seminal plasma; 2) crescendo throughout sperm transit in the female reproductive tract; and 3) are considered to be concluded coincident with sperm responsiveness to acrosome reaction stimulation by zona pellucida ligands (partially modified from original descriptions: Austin, 1951, 1952; Chang, 1951)

Capacitation can be accomplished *in vitro* provided media composition facilitates and supports membrane composition alterations and signal transduction pathway(s) activation that are critical in mimicking that which would occur *in vivo*. It is important to mention that with the exception of scant data on *in vivo* changes in sperm motion (not specifically addressed herein), description of other structural and functional changes occurring *in vivo* and that can be considered as part of or play a role in capacitation remain elusive, at least as they relate to human spermatozoa.

A further complicating issue regarding the detection of changes that are suspected to be a party to capacitation is that spermatozoa can be viewed as tripartite, meaning three compartments. Most experiments investigating capacitation or other pre-fertilization processes assess the cell as a whole, rather than as its separate compartments. Therefore, no distinctions are made between tail, midpiece and head events. For example, if cAMP levels are measured as being elevated during capacitation is this in the head, midpiece and/or tail? Do changes in sperm motion obligate that the cAMP changes are only associated with the motility apparatus?

The purpose of this brief review is to highlight components of culture media whose inclusion has been identified as essential or critical in facilitating the structural and functional changes associated with human sperm capacitation.

Plasma membrane events

The sperm plasma membrane, like that of other cell types, is responsible for the selective trafficking of information between intra- and extracellular spaces (see e.g., Flesch FM, Gadella BM. 2000). Capacitation appears to involve the mobilization and/or removal of certain surface proteins and/or glycoproteins from the sperm plasma membrane, apparently resulting in an increase of membrane fluidity and permeability. These events are followed by or are simultaneous with changes in the lipid composition of the sperm plasma membrane, resulting in: 1) a decrease in net negative surface charge; 2) formation of specialized areas devoid of intramembranous proteins and sterols; and 3) increased concentrations of anionic phospholipids.

Lipid-protein interactions can cause changes in membrane permeability. More specifically, membrane lipids appear to modify the properties of channels that, under normal conditions, maintain an electrochemical balance.

Cholesterol

Changes in lipid composition and distribution within the plasma membrane are early events in capacitation (see e.g., Cross NL 1998). Cholesterol is a major sterol in the sperm plasma membrane and its loss begins soon after sperm are removed from seminal plasma. Cholesterol removal is obligatory for capacitation of human sperm – *in vitro*. The elucidation for this requirement came initially as a result of investigations by Brian Davis and team using rat sperm.

It is thought that cholesterol removal from the sperm plasma membrane alters the cholesterol to phospholipid ratio culminating in an increase in plasma membrane fluidity, which is thought to be necessary for the localization and expression of membrane receptors that will initiate the signaling sequence(s) that culminate in membrane fusion (acrosome reaction).

If cholesterol is not reduced in the plasma membrane then capacitation is inhibited. Evidence for the inhibitory effect of cholesterol comes from experiments where dose-dependent addition of cholesterol to culture medium resulted in a progressive decrease in acrosome reaction inducibility. At a maximal added dose cholesterol prevents the acrosome reaction and fertilization.

In vitro, sperm cholesterol loss is facilitated by the presence of a sterol acceptor molecule in the culture medium, such as serum albumin and fetal cord serum. It has been reported that the type of albumin used for protein supplementation influences the ability of the medium to support capacitation. The reason for the variation is attributed to the presence of a lipid transfer protein (LTP-1) in the albumin. Albumins with high levels of LTP-1 effectively support capacitation whereas removal of LTP-1 diminishes the ability of the albumin to positively affect capacitation.

Cyclodextrin, a cholesterol-depleting complex saccharide molecule, has a high affinity for cholesterol, and that attribute has been useful for investigating and reinforcing the process of membrane cholesterol depletion via sterol receptor molecules. Cyclodextrin can completely substitute for albumin and facilitate the same changes that sperm pass through during culture in albumin-containing media in vitro, reinforcing the view that cholesterol loss is an important determinant of the rate at which sperm become acrosomally responsive.

Ions

The resting potential of the sperm plasma membrane is determined by its permeability to ions. Epididymal fluid composition acts to stabilize membrane polarity by high K^+, and low Na^+ and HCO_3^-. Seminal plasma is hyperosmotic (~320-340 mOsm) relative to serum (~280 mOsm) and this attribute may further contribute to plasma membrane stabilization.

Sperm encounter a shift in membrane stabilizing ions as they migrate out of seminal plasma and into the female reproductive tract secretions whereby K^+ falls and Na^+ and HCO_3^- both rise. Interestingly, the aforementioned serves to cause hyperpolarization of the plasma membrane, which may serve as a priming mechanism for subsequent acrosome reaction responsivity. It is the ionic composition of the female tract, and specifically the oviduct, that has served as the primary template in the development of culture media.

Ca^{2+}

While resident in the epididymis, sperm intracellular concentration of Ca^{2+} is low but that changes when capacitation is initiated, as evidenced by increasing intracellular Ca^{2+} concentrations. While it is agreed that a change in permeability to Ca^{2+} is a part of capacitation it is not known specifically how (mechanism) that change occurs. As it relates to capacitation there is noteworthy ambiguity concerning the specific role and requirement for extracellular Ca^{2+}. It is known that sperm requirements for extracellular Ca^{2+} are dependent upon the process in question, e.g., capacitation, acrosome reaction, hyperactivation. It can also be stated that extracellular calcium is obligatory for intracellular Ca^{2+} concentration changes.

Ca^{2+} -deficient media has been found to inhibit or delay sperm capacitation and, as a consequence, a diminished acrosome reaction. Short-term ($\leq$ 6 hrs) incubation of sperm in a Ca^{2+}-depleted media does not appear to inhibit sperm motility. However, sperm motility is significantly and negatively affected after long-term incubation (>12 hrs). Further,

and importantly, an extracellular lower threshold Ca^{2+} concentration is required for the development of hyperactivation and maximal acrosome reaction in response to intact and solubilized zona.

Recently it was reported that strontium (Sr^{2+}) is as effective as Ca^{2+} in influencing human sperm capacitation-related events, such as protein tyrosine phosphorylation (see below) and hyperactivation, but not for supporting the acrosome reaction.

There is a correlation between protein tyrosine phosphorylation and capacitation in most species evaluated. For example, tyrosine kinase inhibitors, such as genistein, prevent capacitation. It has been thought that protein tyrosine phosphorylation is Ca^{2+} dependent. However, it was recently shown for human and mouse sperm that extracellular Ca^{2+} has a significant negative effect on tyrosine phosphorylation, and by virtue of an associated decrease in intracellular ATP availability.

Bicarbonate (HCO_3^-)

Sperm acquire bicarbonate ion (HCO_3^-) from seminal plasma and once shed from seminal plasma they encounter high concentrations of HCO_3^- in the female reproductive tract. HCO_3^- when added to in vitro culture media as $NaHCO_3$ has been shown to be essential for capacitation. The transmembrane movement of bicarbonate has been associated with an increase in intracellular pH that occurs during capacitation and with the regulation of cAMP, the latter of which also increases during capacitation.

Sperm contain a soluble testicular HCO_3^--dependent adenylate cyclase that is responsible for cAMP synthesis and is stimulated by physiologically relevant HCO_3^- concentrations. HCO_3^- and cAMP stimulate a capacitation-associated increase in protein tyrosine phosphorylation and changes in motility.

Intracellular events

Adenosine 3':5' - cyclic monophosphate (cAMP)
cAMP is an important intracellular second messenger. In sperm it plays an integral role in motility, hyperactivation, protein tyrosine phosphorylation and the acrosome reaction. Intracellular levels of cAMP have been shown to increase with the time-course of in vitro capacitation.

The addition of the cAMP-permeable analogue, dibutyryl cAMP (dbcAMP), to culture media stimulates a time-dependent increase in protein tyrosine phosphorylation. Further, addition of phosphodiesterase inhibitors, compounds that inhibit the breakdown of cAMP thereby maintining high intracellular cAMP concentration, e.g., 3-isobutyl-1-methylxanthine, pentoxyfylline, caffeines, to culture media stimulates a time-dependent increase in protein tyrosine phosphorylation similar to cAMP analgues. Notably, some of the aforementioned compounds have been used in ART as sperm-activators and most typically for ICSI cases where sperm motility is low or absent.

It has been proposed that following production of cAMP, tyrosine phosphorylation occurs through a protein kinase A (PKA)-dependent mechanism, however the mechanism by which PKA signals initiation of tyrosine phosphorylation is currently elusive.

Conclusions

Since the first papers by Austin and Change there have been many subsequent investigations into the processes regulating capacitation. In spite of this tremendous groundswell in information there still remains only one definitive 'marker' for the completion of capacitation, and that is the acrosome reaction. Other 'markers ' of capacitation can be considered as correlative in nature but not defining. One possible explanation for this is that there has been wide variation in the culture conditions under which experiments investigating capacitation have been conducted. For example, a capacitation interval of 3 hrs vs 24 hrs may yield vastly different results concerning intracellular events or membrane structural and permeability changes. As a consequence it becomes difficult to clearly and unequivocally define one or another process as being integral to capacitation.

As this brief outline reflects, culture media that can facilitate sperm capacitation are relatively simple in composition. With that said, this outline also clearly highlights that several components must be present (regardless of media complexity) in order for capacitation to be initiated and optimized so that all subsequent pre-fertilization processes are optimized.

In conclusion, hopefully some of the still enigmatic aspects of capacitation will soon be clarified, coupled with existing literature data so that definitive checkpoints in the capacitation process can be resolved.

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Human sperm capacitation: Clinical aspects

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Learning Objectives

At the end of this presentation the delegate should understand what sperm capacitation involves, what assays can be used to assess capacitation and what media conditions are required for capacitation to take place.

Introduction

When studying capacitation it is clearly important to have a defined endpoint at which a spermatozoon can be considered "capacitated". A series of modified definitions have been developed over time but the only real definition is that capacitated spermatozoa are able to fertilise an oocyte (Chang, 1951; Austin, 1952). Consequently, fertilisation would be the ideal physiological endpoint. However, observing fertilization in a research environment raises many ethical issues and as only a very limited number of human oocytes are available for research, fertilisation of a human oocyte is not a practical endpoint for capacitation. Consequently almost all of the studies on human sperm capacitation have used surrogate markers/definitions for capacitation. This means that only aspects of the fertilising ability of a sperm are being measured e.g. ability to undergo the acrosome reaction or hyperactivate. When studying and subsequently developing new media for supporting capacitation it is clearly necessary to assay for fertilisation as well as surrogate markers.

Assays for capacitation

The most commonly used endpoint of capacitation is acrosome reaction. Acrosome reaction can be induced in spermatozoa by pharmacological agents, such as the calcium ionophore A23187 (Aitken *et al.*, 1993) and lysophosphatidylcholine (de Lamirande and Gagnon, 1993), and more physiological stimuli such as progesterone (Meizel and Turner, 1991) and the zona pellucida (ZP) (Hoshi *et al.*, 1993). An increase in acrosome reaction rates is observed in response to A23187 following incubation in capacitating media but it remains unclear whether pharmacological stimuli force a cell to respond in a non-physiological manner (see Liu and Baker, 1996) and thus these systems may be more suitable for determining damage to a spermatozoon. This is certainly the case for cells exposed to excess ROS where there is a reduced ability to undergo ionophore induced AR because the membranes are severely damaged. Spermatozoa encounter progesterone and ZP during passage through the female reproductive tract and are agonists that could potentially induce acrosome reaction during *in vivo* fertilization and should be considered a favourable endpoint when studying changes that occur during capacitation. Although there is considerable controversy over the nature of the membrane 'receptors' for progesterone and ZP, both require membrane receptors to transduce signals to the cell and thus are more 'physiological'.

The zona-free hamster oocyte sperm penetration assay (SPA) has previously been used to assess sperm capacitation (Barros et al., 1978, 1979). The zona pellucida is required for species specific fertilization; in its absence human spermatozoa can fuse with an oocyte from another species and subsequent nuclear decondensation can be identified (Barros et al., 1978). In order to reduce the variability in the assay, agonists of the AR are often used e.g. progesterone (see Aitken et al., 1993). The assay thus measures AR, fusiogenic ability and decondensation. Controversy exists over the value of the SPA for fertility prediction but performed carefully it does represent a useful assay (see Muller 2000; Oehninger et al., 2000). The main problem is obtaining a source of eggs and clear definition of methods.

The zona-binding assay identifies cells with the ability to adhere to human ZP. It is thought that during capacitation zona-binding proteins become exposed on the surface of the spermatozoa enabling adherence to the ZP and subsequent acrosome reaction (see Overstreet et al., 1980). This assay reproduces physiological interactions but its success greatly depends on the quality of the zona pellucida used. ZP are usually obtained from failed IVF cycles so the ZP have previously come into contact with spermatozoa during patient treatment. The ZP are subsequently stored in a salt solution or frozen which may also have detrimental effects on the structure of the ZP (Matson et al., 1997). Having such potential variability in an assay combined with limited numbers of potential ZP's makes the value of this assay limited. Unfortunately, attempts to produce recombinant human zona proteins have met with limited success and to date no recombinant protein is widely available for diagnostic testing.

The antibiotic chlortetracycline (CTC) has been shown to bind to the surface of spermatozoa. The CTC assay displays four different patterns of fluorescence, one of which appears to be correlated with capacitated spermatozoa (Lee et al., 1987; DasGupta et al., 1993). In mouse spermatozoa, definite results can be identified and so precise conclusions can be made but in the human system CTC binding appears more variable and so the assay is less conclusive. It is possible that with the perceived increase in calcium in capacitated cells more robust systems e.g. fluorimetry can be used to standardise the assay.

Hyperactivation (HA) is often assessed and in some cases used as an indication that a cell is becoming capacitated. Hyperactivation levels certainly increase during incubation in capacitating media (Morales et al., 1988) but it remains uncertain whether hyperactivation is a prerequisite for capacitation or a concomitant process. At least in animals, HA and capacitation can be activated using different pathways (Marquez and Suarez, 2004) and certainly chemical means of inducing HA are not likely to represent capacitated cells. Spermatozoa from different men display variable levels of HA following incubation and often display intermittent periods of HA and so it is difficult to make confident conclusions when only small changes in hyperactivation levels are observed. Physiological induction of HA (using progesterone) may allow maximal stimulation and thus assist in the standardization of the assay. HA is important for fertilization. For example, data from KO studies show that if hyperactivation is prevented sperm are unable to penetrate the ZP and can no longer fertilize (Quill et al., 2003). Data in humans show that failure to undergo HA is indicative of reduced fertilisation success at IVF (Sukcharoen et al., 1995). HA would thus appear to be an assay which, when more standardisation is applied (including better means of assessment e.g. longer tracking) is likely to be a useful tool in studying capacitation.

Which assay should I use?

A clinical assay for human capacitation must assess capacitation in a physiological manner using factors that would be encountered during in vivo or in vitro fertilization. The major difficulty in producing an assay for capacitation is to minimise variability within the system which prevents the use of biological material such as native ZP (Graczykowski et al., 1998). Acrosome reaction in response to a physiological agonist is relatively simple to perform and acrosome-reacted spermatozoa can be clearly identified using immunocytochemistry techniques. Currently, the precise agonist of acrosome reaction in vivo still remains elusive. Progesterone, which is released from the cumulus oophorus (Bar-Ami, 1994), induces acrosome reaction in capacitated spermatozoa but it is unknown whether the levels of progesterone required for this assay are representative of those encountered in the female reproductive tract or during IVF. Ideally artificial ZP could be used to stimulate acrosome reaction but currently full length proteins of correct confirmation have not been synthetically produced. The neoglycoprotein, N-acetyl-a-D-glucosamine (glucNAc) may interact with the putative receptor for ZP3 in human spermatozoa inducing acrosome reaction in capacitated cells (Brandelli, 1997, Brandelli et al., 1994, 1995, 1996). GlucNAc induced acrosome reaction mimics the acrosome reaction induced by ZP glycoproteins (Blackmore and Eisoldt, 1999). This potentially is the best solution minimising variability of the assay system but still inducing acrosome reaction in a physiological manner and so providing a define endpoint for capacitation.

What new assays are on the horizon?

During capacitation an efflux of plasma membrane cholesterol is observed. Merocyanine, a marker for membrane fluidity, has been used successfully to isolate a homogeneous population of boar spermatozoa displaying increased membrane disorder (Flesch et al., 2001). However, in human spermatozoa merocyanine only intercalates into the plasma membrane of dead cells. This is thought to be due to the difference in plasma membrane cholesterol/phospholipid ratio between the species (Cross, 1998) and thus limits the use of this probe for investigating human capacitation. Further research using more sophisticated probes is needed to assess changes in the membrane associated with capacitation.

As we learn more about the biochemical changes concomitant with capacitation (see De Jonge this symposium) more effort needs to be directed into refining these methods for clinical use. For example, considerable progress has been made in the identification of proteins involved in capacitation (see Ficarro, et al., 2003). Whilst such identification is still in its infancy it is likely to provide a very useful source of defined markers. With the development of rapid methods to identify, for example, phosphorylated proteins, such assays may be a future tool in the andrology laboratory.

What needs to be in the media for human sperm to capacitate?

All the information to date suggests three essential ingredients: bicarbonate, calcium and a proteins source. Whilst there are a number of commercial media that support capacitation and thus there is limited value in developing numerous others, there is a clear need to improve the 'physiological' support for sub-optimal cells. For example, sub-optimal spermatozoa have higher levels of ROS damage and thus are likely to benefit from antioxidant protection. Such protection must not be excessive as ROS are necessary for sperm function and thus a delicate balance is be required. As specific defects in the capacitation process have yet to be detailed, primarily because knowledge of the process and its detection remain crude, it is likely that defects do occur and thus specific defined media will need to be developed.

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Metabolic requirements of the early embryo

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Learning Objectives

- To understand why the requirements for early embryos are likely to be relatively simple compared with those for somatic cells.
- To be able to describe factors which determine the requirement of early embryos for oxygen and carbon dioxide
- To know the distinction between endogenous and exogenous nutrients
- To be able to discuss critically the statement: 'Medium X satisfies the needs of the embryo'
- To be able to discuss critically the two main approaches to devising embryo culture media: 'Empirical optimisation' and 'Back to nature'

Lecture summary

The requirements of the early mammalian embryo *in vitro* are relatively simple: a solution of mineral salts, basic nutrients, oxygen/carbon dioxide and a macromolecule such as albumin.

Why?

Early embryos are large, relatively autonomous cells which have barely begun to differentiate and do not have the specialised requirements of somatic (adult) cells.
It is certainly the case that early embryos *in vivo* – in the Fallopian tube and the uterus – will be exposed to complex environments, but we do not know enough to mimic their many constituents adequately and safely *in vitro*. It is also the case that early embryos grow fairly well *in vitro* - for example, over 1 million babies have been born from IVF; a technique which is considered to be basically safe.

The focus of this contribution is on metabolic requirements with a focus on the human embryo. It should be said at the outset, that the present paper should be considered a footnote to the masterly account by Summers and Biggers (2003): *Chemically defined media and the culture of mammalian preimplantation embryos: historical perspective and current issues*, which everyone interested in this subject is urged to read.

Oxygen

The general view, backed up by numerous studies, is that early embryos grow better under 5% rather then 20% oxygen, ie. a pO_2 of around 40 mm Hg rather than 150 mmHg. This is not surprising since the pO_2 in the oviduct of the rhesus monkey, the species closest to the human for which there is good data, is around 40-50 mmHg, depending on cycle stage, decreasing to 10-20 mmHg in the uterus (Fischer and Bavister, 1993). Values for oviduct pO_2 in hamster and rabbit are broadly similar.

It is also generally agreed that having a lower oxygen tension will minimise damage from Reactive Oxygen Species (ROS).

Having said this, it is interesting to speculate on the relationship between oxygen availability, consumption and egg/embryo size.

Using a mathematical approach, Byatt-Smith et al (*Hum Reprod* **6**, 52,1991) showed that while mouse embryos (radius ~ 40) can comfortably satisfy their oxygen requirements when incubated in 5% oxygen, human embryos (radius >60 and therefore about 4x greater volume) are 'on the edge' under 5% oxygen and in danger of becoming marginally hypoxic. This is consistent with the notion that the maximum radius an average mammalian cell can reach is about 60 before oxygen access to the centre of the cell becomes limiting.

These and other considerations lead to discussions about the desirability of mixing/stirring embryos slowly – which undoubtedly occurs in the oviduct and uterus via cilia/muscular activity – to minimise the build-up of unstirred layers around the surface of the cells. For a summary of the latest technology on overcoming the static nature of embryo culture, see the recent view by Wheeler et al (2004).

It also remains the case that many laboratories continue to grow human embryos under 20% oxygen.

Carbon dioxide

Most labs grow embryos in 25 mM HCO_3 under 5% CO_2, which provides a pH of 7.4. To what extent this is desirable is much discussed. One reason for this uncertainty is that the pH in the oviduct lumen is in the alkaline range 7.6-8.2, and measuring HCO_3 concentration is difficult.

Nutrients:

General considerations:

Early embryos can derive nutrients from two sources: exogenously from the culture medium or endogenously. The most likely endogenous source is intracellular fat, especially in the embryos of the large domestic species and the human. There is evidence for the use of triglyceride as an energy source in early cow and pig embryos, but it is a mystery why embryos in some species contain so much fat. One possibility is that fat is required to provide energy for the enormous expansion which occurs after blastocyst formation in the embryos of the domestic species; eg. cows, pigs and sheep.

Macronutrients

The most immediate and quantitatively significant use of macronutrients is to generate ATP; the second, major quantitatively significant need is to provide amino acids for protein synthesis. Amino acids are now added to most embryo culture media and have numerous functions other than as protein constituents, though their quantitative significance of these functions tends not to be considered.

Of the macronutrients, supplementation of culture media with fatty acids has largely been ignored. For example, early embryos *in vitro*, unlike people, do not appear to need a supply of essential fatty acids in their diet. *In vivo*, such nutrients may be provided by uptake of albumin, the major macromolecule in oviduct fluid, by pinocytosis or endocytosis.

Defining requirements for macronutrients:

It is common to see statements like: *'Medium X satisfies the needs of the embryo'*. While such statements may be justified in qualitative terms (i.e., what an embryo needs), they are not justifiable in quantitative terms (i.e., how much of a given nutrient an embryo needs). There are three reasons for this:

1.Information on the quantitites of nutrients consumed *in vitro* – which might indicate how much of a nutrient is required - depend on what is provided to the embryo since the presence of one nutrient(s) can affect the consumption of another.

2. Although, in a few cases, one can define the uptake and metabolic fate of a nutrient quite precisely in kinetic terms (e.g. glucose), it is not possible to translate such data into a value for the concentration of glucose which should be provided in culture media (Leese, 2003).

3. We have little idea what an embryo's 'physiological needs' are, i.e., *in vivo.*

However, it is obviously important to try and define the concentrations of nutrients to which embryos should be exposed. Summers and Biggers (2003) described the two approaches mainly adopted as: (i) 'Let the embryos choose' and (ii) 'Back to nature'. While the 'back to nature' approach is aptly named since it aims to mimic the physiological environment of the embryo, I think the expression 'let the embryos choose' is misleading. It is meant to represent the experimental approach whereby the concentration of one or more culture medium constituents is varied while the concentrations of the other components are kept constant. I would not call this 'let the embryos choose' since embryos are always going to choose what to consume whatever medium they are given, be it a 'back to nature' medium or one devised in some other way, since they have the capacity to adapt to their environment. I would describe the 'let the embryos choose' principle of Summers and Biggers as 'empirical optimisation' i.e., literally: 'resting on trial or experiment' and 'to make as efficient as possible'. One way this principle has been applied is to use data on the consumption of nutrients to make educated guesses on what an embryo requires. Thus, it has long been known that embryos consume oxidative substrates such as pyruvate during early preimplantation development but increase their consumption of glucose as the blastocyst stage is reached and that, at least *in vitro*, much, if not most of this glucose, depending on the species, is converted to lactate. This strongly suggests a 'need' of embryos for glucose during blastocyst formation but does not indicate how much is required.

However, this principle comes unstuck for amino acids, where Houghton et al (2002 *Hum Reprod* **17**, 999-1005 [Corrigendum **18**, 1756-1757])) showed that only a limited number: leucine, serine, arginine, methionine and valine were taken up from a full mixture, by spare cleavage stage human embryos which developed to blastocysts and that only one amino acid; leucine, was consumed at all stages. If the approach: 'give the embryo what it needs', were adopted, the only amino acids added to the culture medium would be leucine, serine, arginine, methionine and valine, but this would be to misinterpret the data on amino acid consumption. Firstly, the term 'consumption' is the net flux of amino acids from the medium and comprises amino acid entry across the plasma membrane, possible exit and re-entry, and metabolic interconversion to other amino acids. Secondly, the pattern is only derived by providing the embryos with a complete mixture of 20 amino acids; if presented singly or as a small group, embryos would most likely consume whatever amino acid (s) was provided.

In its most sophisticated form, 'empirical optimisation' becomes the Simplex Optimisation used successfully by Biggers and his colleagues in the mouse to devise medium SOM and its derivative KSOM (Lawitts and Biggers, 1993). If sufficient embryos were available, it would be possible to apply the technique of Simplex optimisation to the human, however, this is unlikely to be achievable because of the severely limited supply of surplus human embryos. The routes open to formulate human embryo culture media are therefore (i) empirical optimisation using whatever surplus human embryos are available combined with data from animal models and (ii) 'back to nature'. The most widely used animal model of preimplantation development is provided by the mouse. While having great advantages in terms of availability, homogeneity, genetics, successful culture *in vitro* and ease of embryo transfer, it is arguable that closer models to the human, in terms of embryo size, metabolism, time-course of development to the blastocyst stage and onset of zygotic genome activation, are provided by cattle, pig and sheep embryos. What is unfortunate is the practice of trying to optimise the composition of human embryo culture media during routine IVF by using as the 'outcome', the pregnancy rates following embryo transfer. Such an approach ideally needs to be preceded by work on surplus human embryos to help ensure the safety and efficacy of any modifications made .

The 'back to nature' approach attempts to mimic the environment in the Fallopian tube and uterus. The problem then becomes one of obtaining and analysing human tubal fluid and uterine fluid; not an easy task, though accomplished by Borland et al for ions (*J Asst Reprod Gen* **15**, 466, 1998), Gardner et al for pyruvate, glucose and lactate (*Fertil Steril*, **65**, 349, 1996) and indirectly, on Fallopian tubal fluid formed during vascular perfusion, for pyruvate, glucose, lactate and amino acids by Tay et al (*Hum Reprod* **12**, 2451) The best animal models are provided by the cow, pig and sheep, where direct sampling or cannulation *in situ* is possible (eg Kenny et al, *Biol, Reprod* **66**, 1797, 2002).

Growth factors

In the female tract, early embryos will be exposed to a great many (hundreds and possibly, thousands) of growth and related factors, mainly from serum. Embryologists have rightly been reluctant to add such factors singly to human embryo culture media, for fear of precocious or uncontrolled effects. For example, adding a growth factor which is mitogenic may increase development rate and cell number at a time when the embryo should really be increasing apoptosis to overcome cellular stress. There are two ways to approach this problem in a rational way; the first is to analyse human and animal reproductive tract fluids for growth and other factors; and secondly, to supplement culture media with a low concentration of patient's serum, as was widely practiced in the early days of IVF, with as far as is known, no deleterious consequences. The advantage of the second option is that growth factors are likely to be

present in roughly the proportions to which the embryos would be exposed *in vivo*. However, with the move to fully-defined media, option 2 is most unlikely to find favour.

Conclusion

- ✓ We are still some way from producing a medium optimised in terms of metabolic requirements for the culture of early human embryos
- ✓ The 'back to nature' principle offers an approach which is likely to be safe and have acceptable efficiency.
- ✓ It is certainly possible to improve upon nature; this, after all, is what medicine does all the time!

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Culture systems for early embryonic developments

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Introduction

The goal of in-vitro fertilization (IVF) and embryo culture is to provide high quality embryos capable of continued development and implantation, which will result in the birth of healthy babies . Considerable progress has been made in culturing preimplantation embryos since the initial studies were undertaken. Along with other teams, (Tervit et al. 1972), we began to design and define new, more complex culture media in the early seventies, which were based on the composition of genital tract secretions (M n zo 1976). During the initial stages of zygote formation and early cleavage divisions, there is a minimal level of transcription only (Ao et al, 1994), so that at the time of ovulation, the mature oocyte must contain a storage pool of proteins and/or mRNA transcripts in order to maintain its viability and provide the metabolic requirements for fertilization and the first two cleavage divisions of the embryo This stored pool must be capable of coding for all of the enzymes required for metabolic pathways, at the correct equilibrium to support the early stages of embryo development prior to activation of the zygote genome. The cycle during which the zygote genome is activated (Zygotic Gene Activation, ZGA) is the longest cell cycle of preimplantation development: any delay at this time will result in a decrease in the level of mRNA below critical thresholds. Culture conditions have a direct impact on transcription and translation (Ho et al. 1994, Jung 1989,Wrenzycki et al. 1999); the metabolism of the embryo is depressed in vitro (Leese 1995) and protein turnover is accelerated (Jung 1989). A decrease in human embryo viability (Bolton et al. 1989), and cell number (Vlad et al. 1996) has also been observed, all as a result of suboptimal culture conditions. Therefore, for many years embryos were routinely transferred at the so-called “cleavage stages”, as insufficient knowledge was available to allow prolonged successful culture beyond these stages.

Correct timing of blastocyst formation is an initial indication of embryo quality, the ultimate control being live birth rates after transfer. Two solutions have been proposed to satisfy the in vitro requirements that the embryo needs to reach the blastocyst stage: sequential changes in the composition of the culture media, and/or providing a dynamic environment with the help of a cocultured feeder layer of cells. Our first “blastocyst babies” are now 16 years of age, and we present here an overview of human blastocyst culture based upon our experience

Timing of early human preimplantation embryo development

It is now common knowledge that fertilisation occurs very rapidly after insemination. A few hours of oocyte-sperm contact is sufficient to yield the same time course of events that were observed after overnight insemination (M n zo and Barak, 2000). The 4-cell stage is achieved after 40-50 hrs. The cycle of genomic activation begins at this stage and this cell cycle requires a full 24 hours. The “best” embryos usually reach the compacting morula stage (around 32 cells) at 96 hrs pi. On the morning of Day 5, differentiation to blastocyst stage has occurred: the cell number may vary considerably, from 50 cells to 100-120 for early expanding blastocysts. These blastocysts may hatch as early as the morning of D6, but this process is usually observed on D7. One must bear in mind the fact that in vivo hatching is a “dual” process, involving lysis not only by the trophectoderm but also by uterine secretions in the presence of proteolytic enzymes such as chymotrypsin.

Techniques for the culture of human blastocysts *in vitro*

1/ Coculture

The first coculture systems, based upon a postulated autocrine effect were successfully performed with bovine and ovine embryos cocultured with bovine trophoblastic tissue (Camous et al. 1984). Live calves were obtained after transferring embryos that were co-cultured for at least 5 days. Subsequently, we demonstrated that cells of genital origin were not essential for culture of human preimplantation embryos (M n zo et al. 1990), but that success seems to rely on transport epithelia. Coculture with autologous granulosa cells (Freeman et al. 1995), or endometrial epithelial cells (Simon et al. 1999), has been also successful. Coculture on feeder cells may even provide a "better" balance of conditions than are found in the in vivo environment, thus allowing for a selection of embryos with better implantation potential. A study comparing blastocyst transfers with Zygote Intra Fallopian Transfer (ZIFT) allowed us to compare the efficiency of the selection process. Although tubal transfer (ZIFT) supposedly mimics the "best "in vivo conditions, the results did not compare favorably with blastocyst transfer.

	ZIFT	UtB	P
No Transfers	137	217	
No of embryos transferre	356(Z)	448 (Bl)	
Per transfer	2.6	2.06	0.001
Ongoing Pregnancies	39	61	
Percent per transfer	28.5	27.7	NS
Clinical Gest.Sacs	48	86	
Live birth			
(% of embryo transferred)	13.5 (Z)	19.2 (Bl)	0,038

Table 1: Comparison between ZIFT and blastocyst transfers (From M n zo and Janny 1995)
UtB Uterine transfer of blastocysts,
Z : Zygote, Bl : Blastocyst

The co-culture technique differs completely from classical embryo culture technologies. The choice of medium for embryo co-culture studies is critical: it has to satisfy the needs of both the embryo and of the feeder cells. False positive and false negative effects may be immediately linked to the coculture medium. Co-cultured embryos usually have higher cell numbers than do those cultured in simple culture media (Vlad et al. 1996). Randomized experiments (Vlad et al. 1996, Van Blerkom 1993) demonstrated the importance of technical aspects for coculture of human embryos: similar experimental protocols can lead to opposite observations in some circumstances

Interaction with embryo metabolic pathways is an important aspect of feeder cell action**.** Possible adverse situations include deficits in enzyme and messenger mRNA, inhibition of pathways (see phosphofructokinase: Barbehenn et al.1974), and enzyme dis-equilibration (see Glucose phosphate isomerase: M n zo and Khatchadourian 1990). This results in an inability to metabolise some compounds, a waste of energy and an accumulation of superfluous products. Metabolism of feeder cells probably supplies small molecules which allow the cell machinery to function with maximum efficiency. Feeder cells contribute amino acids, leading to a better equilibrium which allows efficient uptake by the embryo Co-culture also provides a favorable Red-Ox potential (equilibrium between reducing and oxidative substances in favor of reducing activity), which is rarely taken into account.

Amino acids may be toxic through their catabolism and the formation of ammonium ions (Gardner and Lane 1993). In open *in vitro* culture conditions, ammonia forms ammonium carbonate and/or bicarbonate, both highly unstable, especially at alkaline pH. Ammonia is eliminated or recycled enzymatically *in vivo* (M n zo et al. 1993, Lane and Gardner 1995), or in co-culture systems, through formation of alanine, glutamine and asparagine.

Macromolecules: No specific embryotrophic factors secreted by feeder layers have as yet been identified.

2/Sequential media

Progress brought about by coculture and the feasibility of blastocyst transfers (M n zo et al.1992a) and freezing (M n zo et al., 1992b) provided the background for investigating the use of cell-free culture conditions (Bertheussen et al. 1996, Gardner et al. 1997, Chouteau et al. 1998) and for the feasibility of blastocysts transfers on a large scale. A first-phase medium must be used from fertilization until the initiation of genomic activation (4-cell stage on day 2), followed by a second-phase medium until D5-6. We recommend rinsing the oocytes 4-5 hrs post insemination, and then moving them to the first phase culture medium. For ICSI, the oocytes should be placed in the first culture medium immediately after injection. Media for culture are usually more "protective" than fertilisation media.

All amino acids are necessary very early after fertilization (M n zo 1976, Gardner and Lane 1996), and even for short-term embryo handling. The use of 5% O2 may increase blastocyst formation. High external concentrations of glucose in an excessively simple culture medium is toxic, rather than the glucose *per se*. The present trend towards removing glucose and phosphate from mammalian embryo culture media is not physiological, and replaces one artefact with another. However,high levels of glucose may have a deleterious effect through an increase in free radical formation. This aspect is particularly obvious in diabetic mammals (Pampfer et al. 2000) and probably in humans. An excess of glucose does induce shifts in sex ratio, towards female in bovine (Jimenez et al. 2003): the female embryo has a more effective antiapoptotic mechanism. Pyruvate, on the other hand, is an interesting compound, in that it acts not only as an energy source, but can also detoxify ammonia in the embryo, through transamination and export of alanine formed as a result. Glycine, which is the most abundant aminoacid in the female genital tract acts synergistically with taurine to improve culture conditions. Glutamine is also needed before genomic activation. Gardner et al. (1997) reported that essential amino acids can be toxic in the first phase of culture. However, we still do not know which amino acids are "essential" for the early embryo: there is no obvious evidence that methionine (supposedly "essential" in animal nutrition), is indeed toxic in the first phase of culture (Table 2). Methionine is a key aminoacid: it incorporated by the human embryo, as well as by mouse embryos (M n zo et al. 1989), and is used for the synthesis of S-adenosyl methionine (SAM), the universal methylation cofactor involved in methylation of proteins, DNA, lipids, etc. Methionyl tRNA initiates all protein syntheses during mRNA translation. DNA *de novo* methylation is essential for early mammalian development (Okano et al. 2000) and is involved in the critical process of gene imprinting. Altered methylation patterns can lead to silencing or induction of specific genes and thus could affect the produced embryos in such a way as to result in the large calf syndrome. Recent publications emphasized aberrant methylation patterns at the two-cell stage in mice when "ordinary" *in vitro* culture was performed (Shi and Haaf, 2002). In humans, an intriguing excess of imprinting anomalies in children conceived by IVF and/or ICSI have been reported (Cox *et al.*, 2002; DeBaun *et al.*, 2003; Maher *et al.*, 2003, Gicquel *et al.* 2003**,** Orstavik *et a*l., 2003**;** Powell, 2003). Methionine shares a common pathway (trans-sulfuration) with cysteine, involved in the synthesis of glutathione, a universal free radical scavenger (and so antiapoptotic) and potent stimulator of early embryogenesis (Lee et al. 2000). *De novo* synthesis inside the embryo is important, as glutathione is not transported into the embryo. Restriction of sulfur free aminoacids does induce apoptosis (Lu *et al.*, 2003, Kang *et al.*, 2002). Moreover, all the sulfur aminoacids are present in the environment of the embryo during preimplantation development *in vivo*.Ammonia is eliminated through transamination with pyruvate and release of Alanine.

Blastocyst development does not require serum in the medium, but so far albumin is found to be necessary.. Albumin is a complex carrier of all kinds of molecules, and especially lipids and albumin can enter the embryo. Blastocyst formation can be obtained with serum albumin and serum fractions (inappropriately labelled synthetic serum substitute, SSS, Desai et al. 1997). Serum is not necessary for IVF culture (M n zo et al. 1984). According to Niemenn and Wreznycki (2000), serum addition in the bovine system seems to affect mRNA content and to be involved in the "large calf syndrome".

Insulin is usually added for the second phase of culture: whether or not it has the same effect as IGF1 is questionable (Lighten et al. 1998). Growth factors may act through a balance between stimulatory and inhibitory effects. It is important to note that Growth Hormone receptor is present in mouse, bovine and human embryos. However it is not obvious that growth factors can work through an internal loop for a type of " auto-stimulation " process. Mouse embryo development is significantly better when embryos are cultured in groups rather than alone. These results suggest an autocrine/paracrine effect but this effect has been denied in human systems. Blastocysts grown in sequential media seem to be less resistant to cryopreservation, or at least, their cryosensitivity seems to be related to the media in which they have been cultured.

Limiting factors

A 45-50 % blastocyst formation rate can be expected in an overall population which includes patients with advanced maternal age, male factor infertility, and repeated failures of implantation. It is now clear that reported rates which exceed this baseline suggest a selected patient population. Several parameters must be taken into account.

Maternal factors: advanced maternal age may impair very early blastocyst formation. There is an initial decline around the age of 30, and a further decline after the age of 40. Blastocyst formation, and then expansion, is impaired with advanced maternal age (Janny and M n zo 1996). However, a good quality blastocyst obtained within the correct time course, has the same implantation potential (around 25-30%) in for all age groups In our practice, advanced maternal age is a favorite indication for blastocyst transfer.

Paternal effects: During the early years of IVF, it was commonly believed that all embryos that developed to Day 2 had an equal chance of achieving a pregnancy. Blastocyst technology has destroyed this assumption, and we now know that compromised sperm considerably reduces blastocyst formation (Janny and M n zo 1994). This has been confirmed by following the results of blastocyst formation after ICSI (Shoukir et al. 1998, M n zo and Barak 2000).

Epigenetic factors: Anomalies of the centrosome (Navarra et al. 1997), defects in "oocyte activating factor", and in DNA condensation and fragmentation (Sakkas et al. 1997, Evenson et al 1999) have considerable deleterious effects. Sperm DNA fragmentation does not impair blastocyst formation but does severely reduce live births post blastocysts transfer.

Cytogenetic problems : in vitro manipulation of gametes increases the risk of cytogenetic anomalies that are responsible for the arrest of least one half of the embryos (Benkhalifa et al. 1996). These anomalies can obviously arise due to both maternal and paternal problems (i.e chromosome abnormalities in gametes, Aran et al. 1999).

Monozygotic Twinning

Monozygotic twinning has been described by Behr et al (2000) as a penalty of blastocyst transfer in 5% of ongoing pregnancies. Zona hardening has been implicated as a mechanism causing embryos to split at the time of hatching. However, another aspect related to culture media can be considered: cells within the Inner Cell Mass appear to be less resistant to disruption than trophoblastic cells. It is possible that in certain hypersensitive embryos, an excessive glucose level in the media may lead to an over-stimulation of apoptosis: the pro-apoptotic effector Bax was found to be increased in mouse blastocysts exposed to hyperglycemia, through free radical formation (see Pampfer 2000). If a linear polarisation of apoptotic cells occurs, the ICM could split **before** hatching, thus leading to a monozygotic twinning (Menezo and Sakkas 2000). In our hands, out of more than 800 pregnancies obtained with coculture (1988-1998), **not one** resulted in monozygotic twins. Indeed, culture conditions rather than prolonged culture time may be responsible for an increase in MZT (Cassuto et al. 2003).

Conclusion

Until we began to understand some of the interactions that take place in the metabolism of early embryos, the empirical approach to comparisons of different culture media formulations was ineffective. Prior to and during culture, it is important to be aware of and to control the interactions of different compounds with each other and with the gas phase. Sequential media are currently effective in human embryo culture, with blastocyst yields similar to those observed for coculture systems. In order to evaluate new techniques and /or new culture media, *in vitro* grown embryos must be transferred to avoid misleading observations based upon morphology alone. In view of early developmental arrests, embryo transfer at early stages is obviously a process which is too blind. The question is no longer whether or not to carry out transfer of embryos at the blastocyst stage but the challenge now focuses instead upon ensuring that the blastocysts are viable and healthy..

The goal of IVF practice should be to transfer embryos at the blastocyst stage, not as an exception, but as a rule. Ultimately this should lead to an improvement in the success of assisted reproductive technologies, with a lesser likelihood of multiple pregnancies. Further research, together with long-term follow-up studies of the babies is still needed in order to ensure that in vitro culture has no adverse effects on the health of the children born.

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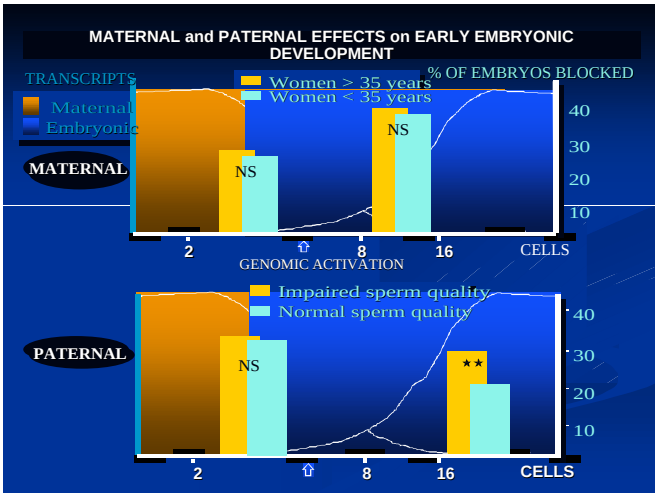
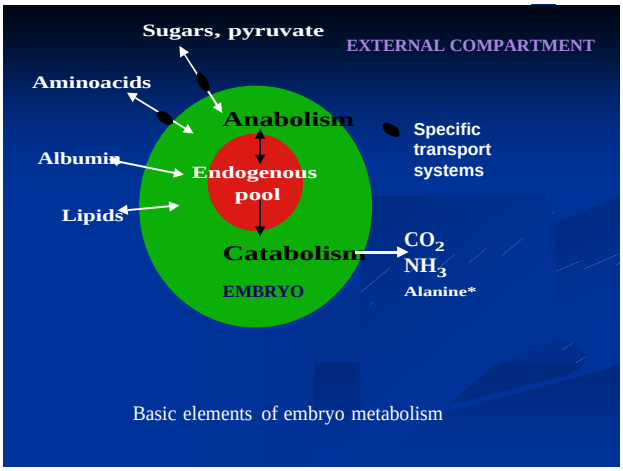
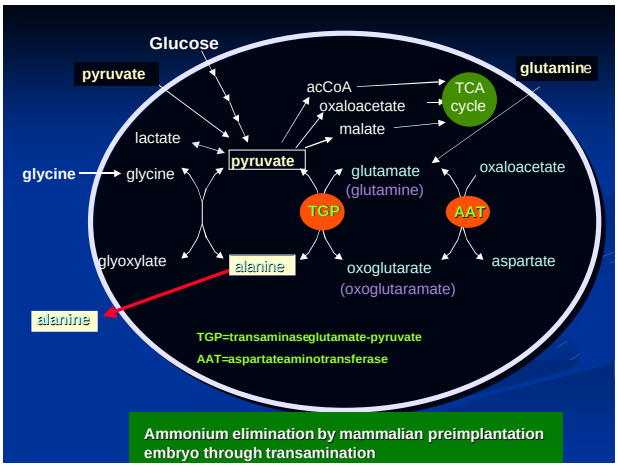
Table 2: Effect of culture in sequential media containing a complete complement of aminoacids in the first and the second phase medium (ISM1/2). Regular IVF after Short insemination time in patients with repeated failures

Patients	186	
Transfers	180 (96%)	
Embryos on D2	1499	
Blastocysts on D5/6	678	(45.2%)
Blastocysts transferred	330	(1.83 per patient)
Pregnancies	86	(47.8% per patient)
Implantation	100	(30.3% per Bl. transferred)

Mean age of the patients: 33.7 Yrs


MATERNAL and PATERNAL EFFECTS on EARLY EMBRYONIC DEVELOPMENT
TRANSCRIPTS
Maternal
Embryonic
MATERNAL
% OF EMBRYOS BLOCKED
Women > 35 years
Women < 35 years
NS
NS
40
30
20
10
2
8
16
CELLS
GENOMIC ACTIVATION
Impaired sperm quality
Normal sperm quality
PATERNAL
NS
★★
40
30
20
10
2
8
16
CELLS


Sugars, pyruvate
EXTERNAL COMPARTMENT
Aminoacids
Anabolism
Specific transport systems
Albumin
Endogenous pool
Lipids
Catabolism
CO_2
NH_3
Alanine*
EMBRYO
Basic elements of embryo metabolism


Glucose
pyruvate
glutamine
acCoA
oxaloacetate
TCA cycle
malate
lactate
pyruvate
glycine
glycine
glutamate
(glutamine)
oxaloacetate
TGP
AAT
glyoxylate
alanine
oxoglutarate
(oxoglutaramate)
aspartate
alanine
TGP=transaminaseglutamate-pyruvate
AAT=aspartateaminotransferase
Ammonium elimination by mammalian preimplantation embryo through transamination

Glucose level in embryo culture medium and apoptosis: Effect of 6hr culture in presence of 20mM of Glucose on apoptosis in murine and bovine blastocysts

Blastocyst cells	Glucose	Cell No	% apoptotic
Murine	0	65	10.7%
	20	67	25 %*
Bovine	0	127	8.2 %
	20	131	20.7 %*

*, p< 0.05 , From Jimenez et al. 2003

Met Uptake and conversion to SAM/SAH in mouse and human embryo
(fmoles/embryo/hr)

	Met Uptake	Conversion
Mouse		
2-Cell	250	9 (3.6%)
Early Morula	350	12 (3.4%)
Compact. Morula	650	33 (5.1%)
Blastocyst	2335	41 (1.8%)
Human		
4-cell	770	26.2 (3.4)

Ménézo et al 1989

Hyperglycemia, Apoptosis and sex ratio Role of X-linked Apoptosis inhibitor (XIAP)

(Bovine blastocyst transfers)

Glucose added (mM)	Blastocysts/ cleaved eggs	Males	Apoptosis (% total cells)
0	27%	52.5%	7.4
10	15.7%*	39%	20.1*
20	9.3%**	35.7%*	37.2**
30	8.2%**	29%*	42.1**

From Jimenez et al. 2003
Culture in SOF medium supplemented with Glucose

SAAD* and cell death
(*Sulfur aminoacid deprivation*)

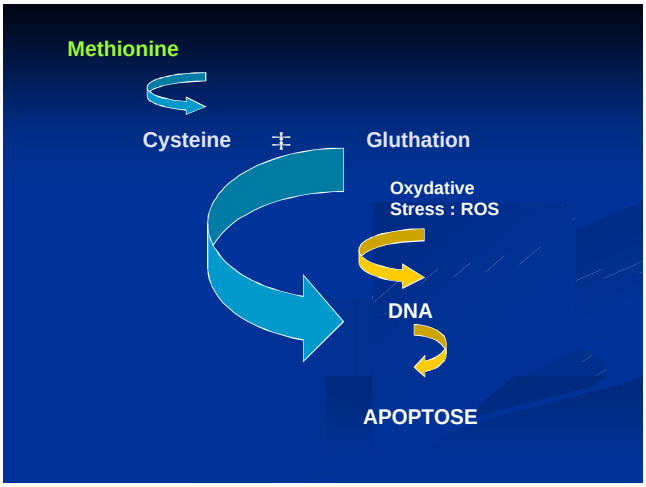
*SAAD increases oxidative stress through a decrease in Glutathion (GSH synthesis)

* It alters Ca++ and free Fe cellular pool

* It induces cell death, independently of caspase activation, via C-Jun-terminal kinase pathway

Most of the first phase Culture media (fertilisation media) have no Sulfur amino acids!!! (… or with a high degree of oxidation: taurine)

Kim HJ and Kim SG Biochem Pharmacol 2002; Kang KW et al. Free Radic Biol Med 2002



Day 5 embryo transfer does not enhance reproductive outcome compared to day 3 transfer using the current culture systems.

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Learning objectives

- To understand the rationale for performing blastocyst transfer

- To be aware of evidence suggesting limitations in the currently used culture media

- To be able to summarize the current literature comparing pregnancy rates between day 3 and day 5 transfer based on evidence from retrospective studies and from randomized controlled trials

- To be aware of the pitfalls present in studies comparing day 3 vs day 5 transfer.

- To be able to advise patients during consultation about the mode of transfer to be performed

Rationale for blastocyst transfer

Culture until the blastocyst stage allows the replacement of embryos which have an activated embryonic genome (Braude et al 1988) and are at a more advanced stage of development compared with cleavage stage embryos. Therefore, it could be expected to increase the probability of implantation. Moreover, it could be associated with a decrease in multiple pregnancy rates by allowing a reduction in the number of replaced embryos without compromising the chance of pregnancy.

However, this requires that the elimination of embryos in extended culture from day 3 to day 5-6 should be dependent solely on their inherited survival potential and not be a consequence of an adverse effect exerted by the sequential media used for culture.

Where this is the case, it is likely that even though blastocyst transfer may select embryos with a greater implantation potential, this will be at the cost of losing embryos that could have implanted if transferred prior to the blastocyst stage. As a consequence by extending embryo culture to day 5 a number of patients who might have become pregnant if embryos had been replaced on day 3 will fail to become pregnant.

Do the currently used culture media compromise the quality of developing blastocysts in vitro?

In patients with poor prognosis (those with no eight-cell embryos on day 3) blastocyst culture and transfer has been reported to result in 0% pregnancy rate in contrast with day 3 transfer which has led to 33.3% ongoing pregnancy rate (Racowsky et al., 2000). This suggests that the uterine environment may rescue some of the embryos that will not survive blastocyst culture (Racowsky et al., 2000).

A reduction in viability of embryos with cleavage abnormalities has also been shown by Alikani et al (2000), who suggested that until culture media are further refined, extended culture should be limited to those embryos with optimal development for the first 3 days in culture.

Does blastocyst transfer enhance pregnancy rate compared with day 3 transfer?

Evidence from retrospective studies

Milki et al. (2000) compared day 5 vs day 3 transfers and showed a significantly higher viable pregnancy rate (68% vs 46%, respectively) and implantation rate (47% vs 20%, respectively) in a small number of patients who had more than three 8-cell embryos on day 3 of culture. More embryos were transferred on day 3 than on day 5 (4.6 vs 2.4, respectively). Multiple pregnancy rates did not differ significantly between the two methods, although the rate was higher in the day 3 group than in the day 5 group (65% vs 44%, respectively).

From a limited number of patients with multiple in-vitro fertilization (IVF) failures and with at least 3 embryos available in the previous cycle, Cruz et al. (1999) concluded that blastocyst transfer leads to a higher clinical pregnancy rate (40%) than does day 3 transfer (9.1%). Moreover, the implantation rate was higher too in the day 5 group than in day 3 group (11.3% vs. 3.4%, respectively), while more embryos were transferred on day 3 than on day 5 (5.4 vs. 3.1, respectively). No multiple pregnancies were observed in the blastocyst group, although the difference with day 3 transfer was not significant.

Racowsky et al. (2000) reported that for patients with at least 8 zygotes after fertilization similar pregnancy rates were observed between day 5 (43.2%) and day 3 (43.9%) transfers. The implantation rate was higher when embryos were replaced on day 5 (31.2% vs 19.5%). Again, more embryos were transferred on day 3 than day 5. No difference in multiple pregnancy rates was observed between the two groups.

Analyzing day 5 vs day 3 transfer in patients with at least five oocytes fertilized Abdelmassih et al (2001) observed a higher ongoing pregnancy rate (41.0% vs 29.4%, respectively) and implantation rate (18.5% vs11.5%, respectively) in the day 5 group. More embryos were transferred in the day 3 group than in the day 5 group (4.6 vs 3, respectively). A higher multiple pregnancy rate was present in the day 3 vs day 5 group (47.1% vs 28.5%, respectively)

Balaban et al. (2001) observed that blastocyst transfer results in a higher implantation rate than does day 3 transfer (15% vs 5.9%, respectively) in patients with only grade 3 and 4 embryos available for transfer on day 3. More embryos were replaced on day 3 than on day 5 (5.2 vs 2.4, respectively). No difference was observed in pregnancy rates (27.2% vs 33.5%, respectively) or multiple pregnancy rates between the two groups (13.6% vs 9.4%, respectively).

Performing blastocyst transfer for all their patients, Marek et al. (1999) demonstrated a higher ongoing pregnancy rate and implantation rate than for patients in whom day 3 transfer was performed prior to extended culture introduction (pregnancy rates: 46.9 % vs 36.9%, respectively; implantation rates: 32.4% vs 23.3%, respectively). Again more embryos were transferred on day 3 (3 vs 2.5). The multiple pregnancy rates were similar in the day 3 and day 5 groups (49% and 42%, respectively).

Wilson et al. (2002) showed a higher ongoing pregnancy rate (52% vs 42%) and implantation rate (43% vs 27%) when comparing day 5 (n=570) with day 3 (n=419) transfers. More embryos were replaced on day 3 (2.7) than on day 5 (2.0). Moreover, no difference was observed in multiple pregnancy rates between the two groups compared (day 3: 36% vs day 5: 34%).

In oocyte recipients, Schoolcraft et al. (2000) reported that blastocyst transfer compared with day 3 transfer results in a higher clinical pregnancy rate (87.6% vs 75.0%, respectively) and implantation rate (65.0% vs 41.6%, respectively). More embryos were replaced on day 3 than on day 5 (3.2 vs 2.1, respectively). Moreover, no difference was observed in multiple pregnancy rates when embryos were transferred on either day 3 (40.5%) or day 5 (44.2%).

To sum up, it appears that retrospective studies consistently support the view that blastocyst transfer results in a higher implantation rate than does day 3 transfer. Results are not always so clear regarding pregnancy rates, which in some studies did not differ between the two methods (Racowsky et al. 2000; Balaban et al., 2001). Interestingly, multiple pregnancy rates were similar in both day 3 and day 5 transfers.

Pitfalls of retrospective studies comparing day3 vs day 5 transfer

Consistently more embryos were transferred on day 3 than on day 5 in all of the above retrospective studies. However, as the number of embryos replaced increases, a “dilution effect” takes place.

If we compare the implantation rates achieved in two exactly similar groups of patients, in whom, however, unequal numbers of embryos are transferred on the same day of culture (e.g day 3), the only case that there will no be no difference in implantation rates is if all the embryos are of similar quality. If however embryo quality is not the same within embryos, as in human IVF, then the chance to transfer embryos of inferior quality increases in parallel with the number of embryos transferred (assuming that we first transfer the best embryos). As a consequence the implantation rate will decrease in the group of patients in which more embryos are transferred. This kind of bias is present in all of the retrospective studies mentioned.

Arguing, therefore, on the basis of retrospective studies, that the higher implantation rate observed with extended culture is a proof that blastocyst transfer is superior to day 3 culture is not justified.

Evidence from randomized controlled trials(RCTs)

Table 1. **Randomized trials comparing day 3 vs day 5 transfer**

Authors	**Selection criteria**	**Culture media**	**Mean number of embryos transferred**		**Pregnancy rate**		**Implantation rate**		**Multiple pregnancy rate**	
no of patients included										
Gardner et al. 1998 [a]	Patients with >10 follicles of > 12mm on day 8 of the cycle	Sequential media	3.7	2.2	66%	71%	30.1 %*	50.5 %	-	-
day 3: 47 , day 5: 45										
Coskun et al. 2000 [b]	All patients who had 4 or more fertilized oocytes	Sequential media	2.3	2.2	39%	39%	21.3 %	23.8 %	33%	38%
day 3: 101 , day 5: 100										
Karaki et al. 2002 [b]	Patients with at least 5 2PN embryos were available	Sequential media	3.5	2.0	26%	29%	12.7 %*	26.1 %	48%	39%
day 3: 82 , day 5: 80										
Levron et al 2002 [c]	Patients with more than five zygotes on day 1	Sequential media	3.1	2.3	45.5 % *	18.6 %	38.7 % *	20.2 %	40%	50%
Day 3 : 44, day 5: 46										
Utsunomiya et al 2002 [d]	All patients with oocyte retrieval	Sequential media	2.9	3.0	26.3 %	24.8 %	11.7 %	9.2%	-	-
Day 3: 184, day 5: 180										
Rienzi et al 2002[a]	<38 years, with at least 8 two-pronucleated zygotes on the day following ICSI	Sequential media	2.0	2.0	56%	58%	35%	38%	-	-
Day 3: 48, day 5: 50										

(* $p<0.05$).

Cumulative delivery rates including both fresh and frozen cycles per oocyte retrieval: day 3: 77% day5: 52%, $p<0.01$

Randomization type: a: computer generated list, b: by sealed envelopes, c: not stated, d: sequence of ovum retrieval.

As it can be observed in Table 1 in none of the 6 randomized controlled trials is pregnancy rate significantly better with blastocyst transfer as compared to day 3 transfer. In the study by Levron et al (2002) blastocyst results in a worse pregnancy rate than day 3 transfer. Moreover, in only 2 out of 6 RCTs is implantation rate better with blastocyst transfer while in the study by Levron et al (2002) is worse than day 3 transfer.

It therefore appears that the published prospective randomized trials do not support the view that day 5 transfer results in a better outcome than day 3 transfer when studies using sequential media for embryo culture are considered.

Do randomized control trials suggest that there is a higher implantation rate in the day 5 as compared to the day three group?

Higher implantation rates for blastocyst transfer were reported in the studies by Gardner et al. (1998) and Karaki et al. (2002). However, at the same time more embryos were transferred on day 3 than on day 5. The validity of comparing implantation rates is therefore questionable, since the denominator is different across the groups compared. If more embryos are transferred in day 3 patients, then a "dilution effect", as mentioned above, is to be expected. Where the numbers of embryos replaced were similar between the two groups, no difference was observed in implantation rates.

Should we advise patients undergoing in-vitro fertilization to start a cycle leading to a day-3 or a day-5 transfer?

Counseling of patients on the mode of embryo transfer based on the results of the above RCTs studies is inevitably conditional. The couple will only know the type of embryo transfer after a certain stimulation day is reached, after oocyte retrieval or after insemination of the oocytes retrieved.

A RCTwas performed at the Centre for Reproductive Medicine of the Dutch-Speaking Brussels Free University from January 2001 until December 2003 (Table 2) with the objective to compare ongoing pregnancy rates per started cycle between patients randomized, prior to initiation of stimulation, to perform embryo replacement either on day-3 or on day-5 of in-vitro culture (Kolibianakis et al, submitted).

Table 2

Outcome of patients randomized prior to initiation of stimulation to start an IVF cycle leading to day 3 or day 5 transfer.

	Day-3 group	**Day-5 group**
Patients who initiated an IVF cycle (n)	**234**	**226**
Patients who reached oocyte retrieval n (%)	227 (97.0%)	215 (95.1%)
Patients with embryo transfer n (%)	218 (93.2%)[a]	190 (84.1%)[a]
Positive hCG n (%)	96 (41.0%)	94 (41.6%)
Biochemical pregnancies n (%)	11 (11.5%)	12 (12.8%)
First trimester miscarriage n (%)	10 (10.4)	6 (6.4)
Extrauterine pregnancy n (%)	0 (0%)	1 (1.1%)
Ongoing pregnancies n	75	75
Ongoing pregnancy rate per started cycle % (95	32.1% (26.4-38.2)	33.2% (27.3-39.5).
Ongoing pregnancy rate per oocyte retriev (95%CI)	33.1% (27.2-39.4)	33.2% (27.3-39.5)
Ongoing pregnancy rate per embryo transf (95%CI)	34.4% (28.4-40.9)	39.5% (32.8-46.5)
Ongoing implantation rate %±SEM	24.5±2.5%	26.6±2.7%
Multiple pregnancy rate % (95%CI)	20 (26.7%)	15 (20.0%)
Proportion of patients with cryopreserved embry	61.5 (144/234)[b]	50.4 (114/226)[b]

[a] $p<0.001$

[b] $p<0.02$

Based on the results of Table 2 it can be concluded that advising patients at consultation to initiate an IVF cycle leading to a day-5 transfer does not appear to increase the probability of ongoing pregnancy compared to day-3 transfer. Moreover, it is associated with a significantly higher chance of not performing embryo transfer and a significantly lower probability of cryopreservation.

In conclusion, although blastocyst culture and transfer is feasible with the use of sequential media randomized controlled trials published to date do not show that it results in a better reproductive outcome than does day 3 transfer.

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Day 5 transfer is the best strategy to reduce the number of embryos transferred in order to avoid multiple pregnancy

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Learning Objectives

- To identify the potential advantages of blastocyst transfer
- To be able to analyze IVF treatment in a holistic fashion
- To evaluate the merits of single embryo transfer

Introduction

The decision regarding what stage to transfer human embryos is a source of great debate. Due to difficulties in maintaining the human embryo in culture for more than a couple of days, cleavage-stage embryo transfer on day 2 or 3 has become routine. The relatively recent introduction of sequential culture media, which can facilitate successful extended culture, has refocused attention upon the role of the human blastocyst in IVF. Discussions on blastocyst culture and transfer have tended to concentrate on culture media and their formulations. However, culture media are only one part of the laboratory system, and the laboratory is one part of IVF treatment. Therefore, in order to optimize extended culture one needs to focus on all aspects of the treatment cycle. For example different stimulation regimens will not only affect embryo quality and metabolism (Hardy et al. 1995), but have a direct impact on endometrial function and therefore uterine receptivity (Simon et al. 1998). Furthermore, factors in the laboratory other than medium composition have a direct effect on embryo development in culture (Gardner and Lane 2003a). It is therefore important to keep such things in mind when comparing results from one program to another. Unfortunately, this means direct comparisons are very difficult to perform and interpretation of data can be difficult. Therefore, should a program establish a benefit from employing extended culture and blastocyst transfer, then one should be prepared to make changes other than culture media in order to mimic their results.

A major compounding variable with regards to data comparisons between clinics are the patients themselves. It has been inferred that those clinics that fare better with extended culture have a “more favorable” population of patients. However, there exists a group of patients who represent as close as possible a ‘gold standard’ in IVF, oocyte donors. It is proposed that this group of patients will let us determine just how efficient IVF in the human can be, and should be used whenever possible in the comparison of different clinics.

Suitability of the uterus for the cleavage stage embryo

It is perhaps serendipitous that the human uterine environment can support the development of the human pronucleate and cleavage stage embryo, as this is in contrast to other mammalian species including non-human primates (Marston et al. 1977). Although the human cleavage stage embryo can develop in the uterus, a region of the human female reproductive tract that it would not normally see until day 4 (Croxatto et al. 1978), the question must be asked, how suitable is such an environment, especially as it follows the exposure of the patient to exogenous gonadotropins. Had the pre-compacted human embryo not exhibited this ability to survive in the uterus then IVF as we know it would not had progressed, rather we would have had to wait until suitable culture conditions were developed capable of supporting blastocyst development.

The question is therefore whether the uterine environment following ovarian hyperstimulation is a more suitable environment for the early human embryo than the laboratory. In all mammalian species studied to date, the transfer of embryos to the uterus prior to compaction (and therefore before the generation of the first transporting epithelium) does not lead to as high pregnancy rates compared to the transfer of morulae or blastocysts. The significance of compaction is considerable as it represents the generation of the first transporting epithelium of the conceptus. Fol-

lowing the formation of this transporting epithelium the embryo can better regulate its intracellular physiology as shown by its increased ability to buffer pHi (Edwards et al. 1998) and to cope with osmotic stress (Hammer et al. 2000). In general, the post compaction mammalian embryo can tolerate a wider range of environments.
However it is evident that pregnancies in the human can be obtained after asynchronous transfers. Like the mammalian embryo's ability to grow in a wide variety of culture conditions (Gardner et al. 2000), the human embryo can adapt to its environment and continue its development. This is termed embryo plasticity. However, this plasticity should not make us complacent and should not be perceived in a positive light, as the more an embryo has to adapt to its environment the more its viability is compromised (Gardner 1998). By placing the human embryo into the uterus before compaction it will expose the embryo to concentrations of carbohydrates (Gardner et al. 1996), amino acids, gases and pH, to which it is not normally exposed to (Maas et al. 1977; Fischer and Bavister 1993). Thus the cleavage stage human embryo will have to adapt its physiology and metabolism in response to the uterine environment.

It is evident from in vitro studies that such adaptation to its environment is not without stress to the embryo (Gardner 1998; Gardner and Lane 1998). Furthermore, when cleavage stage embryos are replaced in the uterus one has to consider that this is not a typical uterine environment, but rather one following hyperstimulation. It has been demonstrated in animal models that the environment within the female tract following gonadotropin treatment is not as supportive to embryonic development as a non-stimulated environment (Van der Auwera et al. 1999; Ertzeid and Storeng 2001) . Clinical data also supports the hypothesis that following hyperstimulation the uterine milieu is compromised (Pellicer et al. 1996). So therefore it may be preferable to expose embryos to such an altered environment for as short a period as possible, and this can be facilitated by blastocyst transfer.

In animal models, embryo expulsion rates decrease as the interval between ovulation and transfer increases. This finding has been attributed to higher progesterone levels that in turn inhibit uterine contractions. Fanchin and colleagues have subsequently determined that uterine junctional zone contractions progressively decrease as one moves farther into the luteal phase, and that fewer uterine contractions are associated with improved pregnancy rates (Fanchin et al. 1998). This therefore presents a further reason for moving to blastocyst transfer.

Suitability of the laboratory for the cleavage stage embryo

There has been a significant increase in our ability to support human embryo development in culture over the past decade (Bavister 1995; Gardner and Lane 1999; Gardner et al. 2000; Pool 2002). This has stemmed from basic research on the embryos of several mammalian species as well as increased research on the human embryo itself. Culture media formulations have received the most attention. However, the media are only a part of an embryo culture system (Gardner and Lane 2003a), and all aspects of this system must be addressed in order to get optimal embryo development. Components of the culture system which have a direct impact on how embryo culture media perform are gas phase, embryo incubation volume and embryo group size, and type of macromolecule supplementation. All of these have been recently reviewed elsewhere and are represented in Figure 1.

It is evident that culture media are now available that can support high levels of human blastocyst development which can subsequently implant at very high rates (around 50%). In the case of oocyte donors, which represent an excellent source of quality oocytes, blastocyst rates of 60% can be routinely obtained (Patton et al. 1999; Schoolcraft and Gardner 2000; Langley et al. 2001) with concomitant implantation rates (fetal heartbeat) of 65%. Such an implantation rate is significantly higher than that obtained for oocyte donors having an embryo transfer on day 3 in the same program (41.6%).

Cleavage stage or blastocysts: Which to transfer?

Huisman et al., (Huisman et al. 1994) in a retrospective analysis, compared day 2, day 3, and day 4 transfers. Overall implantation and pregnancy rates were not significantly different. However, when cavitating morula were available on day 4, an implantation rate of 41% was attained. Without cavitating morula, implantation rates on day 2, day 3 or day 4 range between 12.7% and 14.4%. Scholtes and Zeilmaker (Scholtes and Zeilmaker 1996) performed a randomized trial comparing day 3 versus day 5 embryo transfer. The pregnancy rates were 40% for day 5 transfers compared to 28% for day 3. The implantation rates for day 5 were 23% compared to 14% for day 3. Significantly, both of the above studies used a mixture of Earle's and Ham's F10 media for culture, which cannot be described as sequential even though technically two media were employed. Therefore care must be taken when comparing data when different

types of culture systems were employed, as it is evident that although different culture conditions can support similar rates of blastocyst development, subsequent implantation rates can be dramatically different (Gardner and Lane 1998).

The use of sequential culture systems to support human blastocyst development was initially offered to patients with either a good response to gonadotropins (Gardner et al. 1998) or with >4 eight-cell embryos on day 3 (Milki et al. 2000). This approach for good prognosis patients led to a significant increase in implantation rates and facilitated the establishment of high pregnancy rates (70%) with a concomitant reduction in the number of embryos transferred. Therefore the use of sequential culture media to facilitate blastocyst transfer has led to a significant reduction in the number of high order multiple gestations.

Subsequently, sequential media and blastocyst transfer have been used successfully to treat patients with poor quality embryos (Balaban et al. 2001; Langley et al. 2001), patients with multiple IVF failures (Cruz et al. 1999), patients with low numbers of oocytes and embryos (Wilson et al. 2002) and oocyte donors (Schoolcraft and Gardner 2000). In all of the situations listed extended embryo culture has been associated with an increase in IVF success rates, although a number of reports (Alikani et al. 2000; Coskun et al. 2000; Rienzi et al. 2002) have questioned such benefits.

In support of the move to blastocyst transfer, nine prospective randomized trials on blastocyst transfer following the use of sequential media have been performed (Gardner et al. 1998; Coskun et al. 2000; Karaki et al. 2002; Levron et al. 2002; Utsunomiya et al. 2002; Rienzi et al. 2002; Van der Auwera et al. 2002; Frattarelli et al. 2003; Margreiter M et al. 2003). Five have reported a significant increase in implantation rates when embryos were transferred at the blastocyst stage on day 5 rather than at the cleavage stage. Three of the trials reported no difference in implantation rate with respect to day of transfer, while one clinic reported a lower implantation rate when day 5 transfer was used. From the available data from prospective randomized studies using sequential media, it appears that the balance is in favor of day 5 transfers. Significantly, in a model to determine which patients should have single embryo transfer, it was determined that outcome was more favorable with day 5 than day 3 transfer (Hunault et al. 2002). Furthermore, in a Cochrane review on cleavage stage versus blastocyst stage embryo transfer (Blake et al. 2003) it was concluded that in clinical trials in which sequential media was used there was “a substantial improvement in implantation rates” following blastocyst transfer.

Significance of cryopreservation

The introduction of blastocyst culture was met with much speculation as not all laboratories were able to cryopreserve blastocysts that were not transferred (Alper et al. 2001). However, with the development of more suitable cryopreservation procedures it is now possible to obtain implantation and ongoing pregnancy rates of 30 and 60% respectively using frozen-thawed blastocysts (Gardner et al. 2003; Veeck 2003).

The move to single embryo transfer (SET)

In our experience, moving to blastocyst stage transfer results in an increase in implantation rates. Although we have been successful in significantly reducing high order multiple gestations, the incidence of twins remains 50% when two blastocysts are transferred. Therefore, in an attempt to eliminate multiple gestations completely, we have performed a prospective randomized trial on single blastocyst transfer.

In a population of patients with a day 3 FSH £ 10 mIU/mL and at least 10 follicles >12 mm in diameter on day of hCG administration (age range 26 to 43 years), it was possible to establish an ongoing pregnancy rate of 60.9% without any incidence of twins (Gardner et al. 2004b). Significantly, when two blastocysts were transferred, the pregnancy rate rose to 76% but with a 47.4% incidence of twins.

In a previously reported prospective randomized trial of single embryo transfer (SET) vs. double embryo transfer (DET) on day 3 performed on good prognosis patients (<34 years of age, no previous IVF and who had at least two top quality embryos at the time of embryo transfer) an OPR of 38.5% was attained in the SET group (Gerris et al. 1999). The implantation rate reported for the SET group was 42.3%. Clearly such implantation and pregnancy rates are excellent, but do fall somewhat short of those obtained with the transfer of a single blastocyst. Therefore the move to single blastocyst transfer (SBT) appears a more viable alternative to SET on day 3 in good prognosis patients. A difficulty that arises in comparing data such as these is trying to ascertain the differences in the patient populations,

i.e. are studies biased by patient selection? In a further report of Gerris et al. it is documented that of 1559 oocyte retrievals between the years of 1998-2001, 385 patients had a single embryo transfer (Gerris et al. 2002). This figure represents less than 25% of the general patient population during the time of the study. Using the selection criteria for entry into the current study, 74% of IVF patients attending for infertility treatment and 91% of oocyte donors could elect to have a single blastocyst transfer.

Conclusions

The cost effectiveness of IVF has been analyzed (Collins 2001), and the negative impact of IVF on the community are the heavy costs associated with twin, triplet and quadruplet deliveries. Looking at the cost-effectiveness of a single embryo transfer (SET) compared to a double embryo transfer (DET) on day 3 has revealed that even though more ART cycles are required when employing SET, the overall cost per child born is the same as or cheaper than as DET due to the increased costs associated with multiple births in the latter group (Wolner-Hanssen and Rydhstroem 1998; De Sutter et al. 2002). Therefore, there are no financial reasons not to elect to move to single embryo transfer, except in countries where the patient has to carry the costs of an IVF treatment but where insurance carriers cover delivery and neonatal care.

Blastocyst culture and transfer represents an effective means of eliminating high order multiple gestations in good prognosis patients, and more recently it has been shown that it can be applied to all patients entering an IVF program with a concomitant increase in the efficiency of patient treatment. Certainly blastocyst transfer, with resultant high implantation rates, should be considered for patients electing to have a single embryo transferred. The potential benefits of extended culture and blastocyst transfer are reviewed in Table 1.

However, it would be unwise to suggest that blastocyst culture and transfer represents a panacea for all clinics and all patients. Before extended culture should be considered all aspects of clinical and laboratory procedures need to be optimized, a theme highlighted throughout this syllabus. Should problems exist either in patient stimulation protocols or within the laboratory, extended culture will only exacerbate the situation. Extended culture should first be tried on oocyte donors, or those patients who respond well to gonadotropins. Implantation rates of 40% or greater should readily be obtained in such patients. Should such implantation rates not be attained then a thorough analysis of the laboratory, patients' stimulation and luteal support regimens should be undertaken.

The time has come to focus on oocyte quality (Hardy et al. 2001) and endometrial receptivity (Lessey 2001) along with culture conditions, in other words it is time to consider IVF treatment in a more holistic fashion (Figure 1).

Table 1. Potential benefits of blastocyst transfer

- Embryo selection; ability to identify those embryos with limited, as well as those with the highest developmental potential
- Synchronization of embryonic stage with the female tract; reduces cellular stress on the embryo
- Minimize exposure of embryo to hyperstimulated uterine environment
- Reduction in uterine contractions; reduces chance of embryo being expelled
- Ability to undertake cleavage stage embryo biopsy without the need for cryopreservation when the biopsied blastomere has to be sent to a different locale for analysis
- Assessment of true embryo viability; assessing the embryo post genome activation
- High implantation rates; reduces the need to transfer multiple embryos
- Increased ability to undergo cryopreservation
- Increase in overall efficiency of IVF

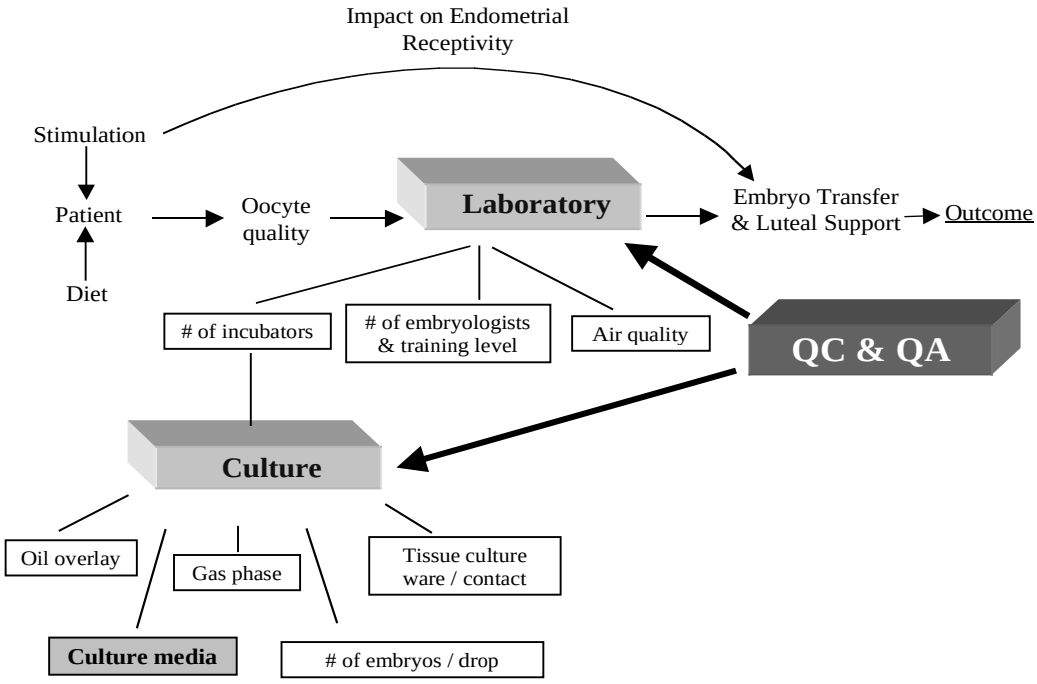

Figure 1. A holistic analysis of human IVF.

This figure serves to illustrate the complex and interdependent nature of human IVF treatment. For example, the stimulation regimen used not only impacts on oocyte quality (and hence embryo physiology and viability (Hardy et al. 1995), but can also affect subsequent endometrial receptivity (Simon et al. 1998; Van der Auwera et al. 1999; Ertzeid and Storeng 2001). Furthermore, the health and dietary status of the patient can have a profound effect on the subsequent developmental capacity of the oocyte and embryo (Kwong et al. 2000; Gardner et al. 2004a). The dietary status of patients attending IVF is typically not considered as a compounding variable, but growing data would indicate otherwise.

In the schematic, the laboratory has been broken down into its core components, only one of which is the culture system. The culture system has in turn been broken down to its components, only one of which is the culture media. Therefore, it would appear rather simplistic to assume that by changing only one part of the culture system (i.e. culture media), that one is going to mimic the results of a given laboratory or clinic.

A major determinant of the success of a laboratory and culture system is the level of quality control and quality assurance in place. For example, one should never assume that anything coming into the laboratory that has not been pre-tested with a relevant bioassay (e.g. mouse embryo assay), is safe merely because a previous lot has performed satisfactorily. Only a small percentage of the contact supplies and tissue culture ware used in IVF comes suitably tested. Therefore it is essential to assume that everything entering the IVF laboratory without a suitable pretest is embryo toxic until proven otherwise.

In our program the 1-cell mouse embryo assay (MEA) is employed to prescreen every lot of tissue culture ware that enters the program, i.e. plastics that are approved for tissue culture. Around 25% of all such material fails the 1-cell MEA (in a simple medium lacking protein after the first 24h). Therefore, if one does not perform QC to this level, 1 in 4 of all contact supplies used clinically will be embryo toxic. In reality many programs cannot allocate the resources required for this level of QC; and when embryo quality is compromised in the laboratory, it is the media that are held responsible, when in fact the lab ware is more often the culprit. For complete details of the conditions for embryo recovery, culture and MEA that are used in our laboratories, the reader may consult the following texts (Gardner and Lane 1999),(Lane and Gardner 2003; Gardner and Lane M 2003b). Modified from an article in Reproductive BioMedicine Online by Gardner DK and Lane M. Towards a single embryo transfer. RBM Online 2003;6:470-481. With permission from Reproductive Healthcare Ltd

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